Host-pathogens interactions during RSV / S. pneumoniae infection immune response and p53 pathway
Daniela Bandeira Brancante Machado

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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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<th>Full Form</th>
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<tbody>
<tr>
<td>C. pneumoniae</td>
<td><em>Chlamyphila pneumoniae</em></td>
</tr>
<tr>
<td>cRNA</td>
<td>complementary RNA</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>F</td>
<td>fusion protein</td>
</tr>
<tr>
<td>G</td>
<td>attachment glycoprotein</td>
</tr>
<tr>
<td>H. influenzae</td>
<td><em>Haemophilus influenzae</em></td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin protein</td>
</tr>
<tr>
<td>hCoV</td>
<td>human Coronaviruses</td>
</tr>
<tr>
<td>HD</td>
<td>hinge domain</td>
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<tr>
<td>hMPV</td>
<td>human Metapneumoviruses</td>
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<td>hPIV</td>
<td>human Parainfluenza viruses</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A viruses</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IP-10</td>
<td>Interferon gamma-induced protein 10</td>
</tr>
<tr>
<td>IRF</td>
<td>interferon regulatory factor</td>
</tr>
<tr>
<td>ISGs</td>
<td>Interferon-stimulated genes</td>
</tr>
<tr>
<td>L</td>
<td>subunit of the RSV-polymerase protein</td>
</tr>
<tr>
<td>M. pneumoniae</td>
<td><em>Mycoplasma pneumoniae</em></td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinase</td>
</tr>
<tr>
<td>Mdm2</td>
<td>mouse double minute 2</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MRSA</td>
<td>methicillin-resistant <em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>N</td>
<td>nucleoprotein</td>
</tr>
<tr>
<td>NA</td>
<td>Neuraminidase protein</td>
</tr>
<tr>
<td>NF-kB</td>
<td>Nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>NHP</td>
<td>Nonhuman Primates</td>
</tr>
<tr>
<td>NLRs</td>
<td>NOD-like receptors</td>
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<td>NP</td>
<td>Nucleoprotein</td>
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<tr>
<td>NS</td>
<td>non-structural protein</td>
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<tr>
<td>NTHi</td>
<td>nonencapsulated strains of <em>H. influenzae</em></td>
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<tr>
<td>P</td>
<td>phosphoprotein</td>
</tr>
<tr>
<td>PA</td>
<td>acid polymerase protein</td>
</tr>
<tr>
<td>PAMPs</td>
<td>pathogen-associated molecular patterns</td>
</tr>
<tr>
<td>PB1</td>
<td>basic polymerase 1 protein</td>
</tr>
<tr>
<td>PB2</td>
<td>basic polymerase 2 protein</td>
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<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PCT</td>
<td>procalcitonin</td>
</tr>
<tr>
<td>PCV</td>
<td>Pneumococcus conjugated vaccine</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Explanation</td>
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<tr>
<td>PRD</td>
<td>proline-rich domain</td>
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<tr>
<td>PRRs</td>
<td>pattern recognition receptors</td>
</tr>
<tr>
<td>RLRs</td>
<td>RIG-I-like receptors</td>
</tr>
<tr>
<td>RNPs</td>
<td>Ribonucleoprotein complexes</td>
</tr>
<tr>
<td>RSV</td>
<td>human Respiratory syncytial viruses</td>
</tr>
<tr>
<td>S. aureus</td>
<td><em>Staphylococcus aureus</em></td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td><em>Streptococcus pneumoniae</em></td>
</tr>
<tr>
<td>SH</td>
<td>small hydrophobic protein</td>
</tr>
<tr>
<td>TA</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>Th</td>
<td>helper T cells type</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>TRAIL</td>
<td>TNF-related apoptosis-inducing ligand</td>
</tr>
<tr>
<td>vRNA</td>
<td>viral RNA</td>
</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
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</table>
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 pediatric et les estimations sont que ce virus cause 3 million d’hospitalisation par an. Un aspect important du pronostic des infections virales est le rôle de co-infection bactérienne. La combinaison d’agents vitaux 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 a cette co-infection mixte dans le macrophages et des modifications dans la réponse immunitaire que nous a permis de mieux comprendre les mécanismes de pathogénèse du VRS dans les cellules épithélial 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 immune ; 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
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.](image)

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 bronchi 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 Coronavirus (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 cytoplasm 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.](image)

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**

*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].
b) *Haemophilus influenzae*

*Haemophilus influenza* (*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 *Clamidophyla 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].

*Chlamydophila pneumoniae* (*C. pneumoniae*), with the two others 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 coinfection 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 enhance 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 sites 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 effectiveness 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 coverture [34, 233]. Even if this Influenza vaccine has a great coverture 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 vaccin 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 coverture of each conjugated vaccine is shown in Table 1.

<table>
<thead>
<tr>
<th>Pneumococcal vaccine</th>
<th>Serotypes</th>
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<tbody>
<tr>
<td>PCV 7</td>
<td>4, 6B, 9V, 14, 18C, 19F, 23F</td>
</tr>
<tr>
<td>PCV 10</td>
<td>1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, 23F</td>
</tr>
<tr>
<td>PCV 13</td>
<td>1, 3, 4, 5, 6B, 6C, 7F, 9V, 14, 18C, 19C, 19F, 23F</td>
</tr>
</tbody>
</table>

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.
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) exists before the invading microorganism, they are effectors cells capable of recognized 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+ T cells are capable to identify and kill infected cells while CD4+ T cells increase macrophages quantity and/or increases 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.
<table>
<thead>
<tr>
<th>TLR</th>
<th>Microbial ligands</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>TLR1</td>
<td>Recognize lipoproteins; minority role in pneumococcus infection.</td>
<td>[276]</td>
</tr>
<tr>
<td>TLR2</td>
<td>Recognizes a large number of binders, such as bacterial peptidoglycans and lipoproteins, and surface viral proteins of RSV and IAV.</td>
<td>[280, 281]</td>
</tr>
<tr>
<td>TLR3</td>
<td>Viral recognition due double-stranded RNA viral of RSV and IAV.</td>
<td>[280-288]</td>
</tr>
<tr>
<td>TLR4</td>
<td>Viral recognition due to surface viral proteins of RSV and IAV; and bacterial lipopolysaccharide and pneumolysin.</td>
<td>[276, 280, 281, 289]</td>
</tr>
<tr>
<td>TLR5</td>
<td>Recognizes gram-negative bacteria and flagellin</td>
<td>[290]</td>
</tr>
<tr>
<td>TLR6</td>
<td>Recognize lipoproteins; minority role in pneumococcus infection</td>
<td>[276]</td>
</tr>
<tr>
<td>TLR7</td>
<td>Viral recognition due single-stranded RNA viral of RSV and IAV</td>
<td>[280-283, 291, 292]</td>
</tr>
<tr>
<td>TLR8</td>
<td>Recognizes single-stranded and double-stranded RNA; relevance during virus infection is unknown.</td>
<td>[293]</td>
</tr>
<tr>
<td>TLR9</td>
<td>Recognizes DNA; protector role against pneumococcus infection</td>
<td>[276, 294]</td>
</tr>
<tr>
<td>TLR1/TLR2</td>
<td>Recognizes large number of bacterial binds, and a large range of viruses</td>
<td>[295]</td>
</tr>
<tr>
<td>TLR2/TLR6</td>
<td>Recognizes lipopolysaccharides and pneumolysin.</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2 TLR and microbial ligands.**

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

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-κB) 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-κB, 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.](image)

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 specifics 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\(^+\) or cytotoxic, kill infected cells while the second class, the CD4\(^+\) or helper, coordinate the immune response promoting the activation of B lymphocytes and macrophages.

CD8\(^+\) 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\(^+\) 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-κB 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-κB-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-kb 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-kB pathway**

The NF-kB family of transcription factors consists of five protein subunits that share an affinity for the kB DNA sequence motif and are divided into two classes: the NF-kB proteins (p105/p50 or NFkB1 and p100/p52 or NFkB2) 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-kb 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-β and IFN-λ1 are predominantly activated by IRF 3, while IRF 7 promotes the transcription of IFN-α and IFN-λ2 and 3 [334, 335]. During RSV infection, IRF3 degradation is promoted, reducing drastically IFN-β 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].

<table>
<thead>
<tr>
<th>ISG</th>
<th>Virus</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Eif2ak2 / Pkr, Oas1b, Rnasel</em></td>
<td>Vesicular stomatitis virus, Vaccinia virus, flavivirus</td>
</tr>
<tr>
<td><em>Isg15</em></td>
<td>Chikungunya Virus, Influenza virus, Vaccinia virus</td>
</tr>
<tr>
<td><em>Ifit1</em></td>
<td>West Nile virus, HCoV</td>
</tr>
<tr>
<td><em>Ifit2</em></td>
<td>Vesicular stomatitis virus, West Nile virus</td>
</tr>
<tr>
<td><em>Ifitm3 / Ifitm</em></td>
<td>Influenza virus, RSV</td>
</tr>
<tr>
<td><em>Rsad2 / viperin</em></td>
<td>West Nile virus, Chikungunya virus</td>
</tr>
<tr>
<td><em>Samhd1</em></td>
<td>HIV</td>
</tr>
<tr>
<td><em>Bst2 / tetherin</em></td>
<td>Vesicular stomatitis virus</td>
</tr>
</tbody>
</table>

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].
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 Kastenhuber 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.
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 marks 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 repress 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 lead 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, can activate p53. Also, some lysine residues of p53 that are modified by acetylation can also be methylated [386-390]. Others 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].

P53α is the canonical full-length p53 protein and inclusion of alternative exons gives rise to p53β and p53γ 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 p53β was described to modulates p53α 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.

![Schematic of the p53 isoforms.](image)

**Figure 16 Schematic of the p53 isoforms.**
p53α 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 Jor Ruiz 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-kB, is known to have opposite effects with p53. Some studies show that this balance NF-kB / p53 coregulates proinflammatory cytokine secretion [316]. Thus, NF-kB activity often shows a negative correlation with p53 while p53 can also suppress the NF-kB pathway.

Figure 18 p53 and NF-kB modulation. During extrinsic or intrinsic stress, p53 and NF-kB 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.
<table>
<thead>
<tr>
<th>Family</th>
<th>Virus</th>
<th>Viral protein</th>
<th>Interplay with p53</th>
<th>Functional effects</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bornaviridae</td>
<td>Borna disease virus</td>
<td>p24</td>
<td>P53 inhibition</td>
<td>Cellular response modification mediated by p53</td>
<td>[429]</td>
</tr>
<tr>
<td>Bunyaviridae</td>
<td>Hantaan virus</td>
<td>N</td>
<td>P53 degradation</td>
<td>Apoptosis prevention</td>
<td>[430]</td>
</tr>
<tr>
<td>Coronaviridae</td>
<td>SARS-CoV</td>
<td>-</td>
<td>P53 down-regulation</td>
<td>Inhibition of antiviral activity by p53</td>
<td>[431]</td>
</tr>
<tr>
<td>Flaviviridae</td>
<td>Zika virus</td>
<td>-</td>
<td>P53 activation</td>
<td>Apoptosis induction</td>
<td>[432]</td>
</tr>
<tr>
<td>Paramyxoviridae</td>
<td>hPIV-3</td>
<td>-</td>
<td>Induced down-regulation of p53</td>
<td>Apoptosis prevention</td>
<td>[433]</td>
</tr>
<tr>
<td>Picornaviridae</td>
<td>Poliovirus</td>
<td>-</td>
<td>P53 degradation</td>
<td>Inhibition of antiviral activity by p53</td>
<td>[434]</td>
</tr>
<tr>
<td>Poxviridae</td>
<td>Vaccinia virus</td>
<td>B1R</td>
<td>P53 phosphorylation</td>
<td>Cellular response modification mediated by p53</td>
<td>[435]</td>
</tr>
<tr>
<td>Reoviridae</td>
<td>Avian reovirus</td>
<td>p17</td>
<td>P53 phosphorylation</td>
<td>Apoptosis induction</td>
<td>[436]</td>
</tr>
<tr>
<td>Retroviridae</td>
<td>HIV Vif</td>
<td>Inhibition of Mdm2-p53 interaction</td>
<td>Cell cycle arrest</td>
<td></td>
<td>[437]</td>
</tr>
<tr>
<td>Rhadboviridae</td>
<td>VSV</td>
<td>-</td>
<td>P53 phosphorylation/ inhibition of p53 acetylation</td>
<td>Apoptosis induction / Inhibition of antiviral response</td>
<td>[438, 439]</td>
</tr>
</tbody>
</table>

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 Δ133p53α and p53β at both transcriptional and posttranscriptional levels. The modulation of Δ133p53α and p53β 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 interacts with p53 protein and inhibit transcriptional activity during infection. Also, an NS1 protein of RSV is capable to inhibit p53 by Mdm2 interaction.
OBJECTIVE
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
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 visions à approfondir la connaissance de la co-infection dans des mécanismes importants pour la cellule, visant à corréler 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 Pathogènes Émergents (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

Innate Immunity response
Chapter 1

Mixed infection
IAV or RSV + S. pneumoniae

In vivo responses
Chapter 3

Cytokines
Chemokines expression

p53 pathway

Respiratory epithelial cells
Chapter 2
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 increases 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
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, macrophage, mRNA and cytokine/chemokine secretion profiles were investigated for human macrophages infected in vitro with influenza virus A/H1N1 and Streptococcus pneumoniae. We observed that in vitro co-infection synergistically increased interferon-γ-induced protein-10 (CXCL10) 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, immunomodulator levels were evaluated in samples from 74 children <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 < 0.01), 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. Streptococcus pneumoniae (SP) is the main causative agent of pneumonia; however, concomitant viral infection occurs in up to 60% of cases of CAP. Clinical data suggest mixed infections are related to a higher severity of inflammatory disease and especially in secondary pneumococcal infection following influenza virus (IAV) infection. - and mixed infection represents a relevant risk factor for pediatric intensive care hospitalization. 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-γ (IFN-γ)-induced protein 10 (IP-10/CXCL10) appears to contribute to the

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pathogenic of several diseases and has been suggested as a potential biomarker of viral infection\(^{11,33}\), late-onset bacterial infection in premature infants\(^4,7\), and a promising biomarker of sepsis and septic shock\(^{12,13}\). Combined analysis of IP-10 and IFN-γ has also been reported as a useful biomarker for diagnosis and monitoring therapeutic efficacy in patients with active tuberculosis\(^{14,15}\) and both remain elevated in the urine of patients with pulmonary diseases in the absence of renal dysfunction\(^{16}\).

With airway epithelial cells\(^1\), resident alveolar macrophages (AMs) and blood monocyte-derived macrophages (recruited into tissues under inflammatory conditions\(^{17,18}\)) represent a major line of defense against both pneumococcal (through their high phagocytic capacity\(^{19,20}\)) and influenza infection\(^{21,22}\). 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 miRNAs) may function as immunomodulators by regulating several pivotal intracellular processes, such as the innate immune response\(^{23-25}\) and antiviral activity\(^{26-28}\); both of these processes are closely related to cell-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 monocytic-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 mRNA 20sA-IAK-STAT/IDOCS-6 regulatory pathway. Subsequently in a retrospective analysis of clinical samples collected from children 5–35 years of age, we confirmed that serum IP-10 level could be related to both viral and/or bacterial infections and disease severity.

Results

Characteristics of MDMs infected by IAV and/or SP. Initially, we investigated the in vitro 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 alone increased even over time after single infection with IAV and correlated with increased production of neutralizing-strain IAV RNA. Maximal 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 6 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 6 h post-infection due to the bacterial impact on cell viability within human macrophages (after 24 h, data not shown). Thirdly, we evaluated the impact of single and mixed infection with IAV and/or SP on MDM viability. Mixed infection significantly decreased cell viability (Fig. 1d) and total cell death at 48 hours post-infection (P < 0.001) compared to single SP infection (50 ± 6%) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection) and 74 ± 4% (SP and IAV infection), respectively (Fig. 1d). Taken together, these results confirmed human MDMs are permissive to mixed viral and bacterial infection.

miRNA, microRNA and protein expression profiling reveal a mixed infection 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 examined 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-κB and TLR signaling pathways (indeed, all interferon-stimulated genes (ISGs) screened, including CXCL10 (fold-change [FC]: 244.9), CCL2 (FC: 34.3) and MX1 (FC: 111.6) were upregulated following mixed infection compared to untreated cells, most of which are closely related to the JSTAT (FC: 52.3), TLR7 (FC: 6.8) and TNFR (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. 3b and Table S2. miRNA 20sA-3p was overexpressed after both single IAV (FC: 6.9), single SP (FC: 1.7) 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. Similarly, miRNA 20sA-3p dysregulation profiles were obtained following IAV and/or SP infections of human macrophages-like (THP-1: monocytic-derived macrophages) or primary MDMs (data not shown).

Thirdly, the accreted levels of various antiviral, pro-inflammatory and immunomodulatory cytokines/chemokines were assessed in IAV- and SP-infected primary MDMs and primary MDMs cell supernatants. We observed a remarkable correlation between the miRNA and protein expression profiles of single or mixed infected MDMs especially regarding CXCL10 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 ± 16,484 pg ml⁻¹) compared to single IAV infection (1,439 ± 566.5 pg ml⁻¹) and single SP infection (4,472 ± 2,901 pg ml⁻¹; P < 0.05; Fig. 2d) 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. 3a). Interestingly, a significant production of IP-10 was also observed in supernatants of primary human airway epithelial cells (HAEIC) mixed infected by IAV and SP compared to the single infections (Fig. 2c). Taken together, the miRNA 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-20a-3p was the most
Figure 1. Single and mixed infection of human macrophage-derived macrophages (MDMs) by IAV and SR.
(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/PR/8/34 hemagglutinin (HA) gene was amplified by real-time qRT-PCR.
(b) Cytotoxicity assay was performed using the proliferation of single IAV-infected MDMs or IAV-infected MDMs following subsequent challenge with SR. (c) Confocal imaging of IAV and SR in mixed-infected MDMs at 3 hours post-IAV infection and 4 hours post-SR infection. DAPI, nuclear stain; blue. IAV, Influenza A virus nucleoprotein stain, green. SP, pseudorabies virus, red. Scale bar = 5 μm. (d) Impact of single and mixed IAV and SR 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.

Overexpression in IAV/SR 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 induction following mixed IAV and SR infection of human MDMs. Using a specific Taqman probe array targeting miR-200a-3p, we confirmed a significant up-regulation of miR-200a-3p following mixed IAV and SR infection of human MDMs (Fig. 1a). In this experiment, a more marked up-regulation of miR-200a-3p was observed following IAV + SR 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 primers 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 infections (Fig. 2a), we hypothesized microRNA-200a-3p may play a role in the regulation of CXCL10 (IFN-β), which was also synergistically upregulated in mixed-infected MDMs (Fig. 2c) and primary human MDMs (Fig. 2a). To test this hypothesis, we investigated the effects of overexpressing (MIM-200a) or inhibiting (UNI-200a) microRNA-200a-3p in THP-1 MDMs. As expected, TGF-β1 miRNA was significantly downregulated in MIM-200a-transfected MDMs (FC = 0.57) and upregulated in UNI-200a-transfected MDMs (FC = 1.70) compared to mock-transfected cells (Fig. 3c). Overexpressing miR-200a-3p (MIM-200a), MIM-200a, and mixed IAV/SR-infected cells enhanced the levels of CXCL10 at 24 h post-infection, whereas partial inhibition (UNI-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 HAECS. (a,b) THP-1 MDMs were infected with IAV (MOI = 1) for 4 h before SP (MOI = 1) was added. Total cellular mRNA (a) or miRNA (b) were extracted 24 h post-infection, reverse-transcribed and amplified using specific RTqPCR Arrays. Major differentially expressed mRNA or miRNAs are shown. (c) THP-1 MDMs, primary human MDMs or primary airway epithelial cells (HAECS) were infected with IAV (MOI = 1) for 4 h before SP (MOI = 1) was added. The concentration of IL-10 in cell supernatant (24 h post-infection) was determined by a multiplex immunoassay on a Luminex platform. Values represent mean ± 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.

CZECH10 [Fig. S4]. These results suggested that R-200a-3p indirectly regulates CZECH10 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 SF. (a) Preceding IAV infection elevates miR-200a-3p expression after mixed infection with SF. MDMs were infected with IAV for 4 hours, then infected with SF and incubated for 24 h, and total endogenous mRNAs were purified. A specific PCR-probe assay targeting miR-200a-3p was used to assess the fold change 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'UTR of both FGFR1 and SOCS6, (c) FGFR1, (d) CXCL10, (e) and (f) SOCS6 expression profiles in MDMs transfected with negative transfection control (NTC), miR-200a mimic (MM-200a) or miR-200a inhibitor (NH-200a). At 18 h after transfection, the MDMs were singly or mixed infected as described previously. At 8 h post-IAV and/or SF infection, total miRNA 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 Dunnet post-hoc test) for data presented in (a, b). 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 SOCS4, SOCS3, IAV nucleoprotein (NP) and β-actin expression in MDMs transfected with negative transfection control (NTC), miR-200a mimic (MM-200a) or miR-200a inhibitor (NH-200a), cultured for 8 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 IV and SI infected human MDMs; therefore, we hypothesized that mir-200a-3p directly regulates a target of the JAK-STAT signaling pathway. Predictive target analysis indicated that the 3 UTR of SOCS6 is targeted by mir-200a-3p (Fig. 3b). SOCS6 proteins are 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 MIR-200a downregulated SOCS6 (PC = 0.57) while inhibition of mir-200a-3p (UNI-200a) upregulated SOCS6 (PC = 1.35), confirming that mir-200a-3p effectively regulates the expression of SOCS6 (Fig. 3c). Moreover, SOCS6 was synergistically downregulated in IV or IV/SP infected MDMs overexpressing miRNA-200a (Fig. 3d), suggesting that both infection and mir-200a-3p negatively regulate the expression of SOCS6. Finally, western blotting confirmed that expression of SOCS6 is sharply reduced following infection, especially after mixed IV and SP infection (Fig. 3g). These results indicate that 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 IV and IP infection of human MDMs and HAEI induced significant production of IP-10. As blood leukocytes and respiratory tract epithelial cells actively contribute to inflammation during pneumonia, we hypothesized that the level of IP-10 in serum of patient with pneumonia may be both indicative of mixed respiratory infection and disease severity. In 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 males, 30 females) were included in this pilot evaluation. According to WHO guidelines, retrospective analysis indicated 42 (56.8%) children had clinical signs of non-severe pneumonia and 32 (43.2%) children had signs of severe pneumonia. The main patient characteristics at inclusion are shown in Table 1. Patients with severe pneumonia had a significantly more recorded episode of dyspnea (P < 0.001), cyanosis (P = 0.03), lower chest inlating (P < 0.001), dailiness to cyanosis (P < 0.001) and enthargy (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 = 0.01). 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-10, IL-9 and MMP-5 were significantly more elevated in serum samples from critically ill patients. Patients with severe pneumonia had significantly higher (4.5±0.3) serum IP-10 levels than those with non-severe pneumonia (4.0±0.1), 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 3. Briefly, in this study, the optimal IP-10 cut-off value for identifying patients with severe pneumonia was 4.240 pg/mL, 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% and specificity of 63.0% and a positive likelihood ratio of 1.74. Prognostic values of IP-10 were closed to pentoitinin (PCT, AUC = 0.70 95% CI 0.58 to 0.82, P < 0.001) and IL-6 (AUC = 0.70 95% CI 0.58 to 0.83, P < 0.001).

IP-10 is significantly associated with viral and bacterial co-infection and pneumonia severity. Multiple PCR-based screening of respiratory and blood samples revealed a high variety of pathogens associations (Table 3). Respiratory viruses were detected in the naso-aspirate (NA) of 63/74 patients (85.1%). Bacterial bacteria of pneumonia (P. aeruginosa, n = 39; S. aureus, n = 1; or H. influenzae type B, n = 7) were identified via real-time PCR in the blood samples of 77 (36.5%) of the patients. Multiple PCR assays allowed the identification of respiratory bacteria in the blood of 19 patients. None of the blood cultures resulted. Among the 74 cases PCR positive for respiratory pathogens, a single virus or bacteria were detected in the NA of 71 (96.6%) and 3 (4.0%) patients, respectively. These 17 (23.3%) cases were defined as the single infection group. The mixed infection group included the 51 (3.3%) cases in which (1) multiple viruses and/or bacteria were identified in NA (58.7%) or 11 (1.5%) without any bacteria identified in blood sample or (2) one or more viruses and/or bacteria were identified in NA and associated with a blood bacteria (24/74; 32.4%). We evaluated whether IP-10 serum level could correlate with viral and bacterial etiologies of pneumonia. Patients with mixed infection had significant highest (5.9-fold IP-10 serum levels 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 infection group) and severe pneumonia (14,427±19,110), compared to only respiratory-identified patients (14,427±19,110, P = 0.081). In detail, a remarkable IP-10 serum level (14,427±19,110) representing 33-fold higher above cut-off value predicting pneumonia severity was observed in patient with SIV in NA co-infected with P. pneumoniae (serotype 14) in pleural effusion and blood. In concordance with our in vitro model of co-infection, the blood samples of 39 (95.38% pg/mL−1) was quantified in blood sample of patient with severe bacterial pneumococcal (serotype 14) pneumonia with 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 disease and related to pneumonia pathogenesis.

Discussion
This study provide 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
<table>
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<th>Patient characteristics</th>
<th>SIRS-SHOCK PNEUMONIA n=64</th>
<th>SEVERE PNEUMONIA n=30</th>
<th>P</th>
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<tr>
<td>Age, months, median (Q1-Q4)</td>
<td>13.5 (8–26.7)</td>
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<td>Weight, kg, median (Q1-Q3)</td>
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<td>Gender, male</td>
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**Clinical examination**

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<td>Axillary temperature, °C, median (Q1-Q3)</td>
<td>37.6 (36.8–38.3)</td>
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<td>Respiratory rate, cycles/min, median (Q1-Q3)</td>
<td>49.5 (44.1–60.7)</td>
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<td>Cardiac rate, cycles/min, median (Q1-Q3)</td>
<td>165.5 (157.8–163.4)</td>
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<td>Nociceptor, %, median (Q1-Q3)</td>
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<td>Oxygen saturation, %, median (Q1-Q3)</td>
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**Coagulation**

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**Radiologic interpretation**

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<tr>
<td>Pleural effusion with parenchymal infiltrate, n (%)</td>
<td>4 (6.3)</td>
<td>15 (50.0)</td>
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<td>Pleural effusion without parenchymal infiltrate, n (%)</td>
<td>1 (1.6)</td>
<td>3 (10.0)</td>
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<td>Geriatric dense homogenous opacification, n (%)</td>
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<td>Localized dense homogenous opacification, n (%)</td>
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<td>Interstitial pneumonitis, n (%)</td>
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<td>Pneumothorax, n (%)</td>
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**Medical history**

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<th>SEVERE PNEUMONIA n=30</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Past hospitalization, n (%)</td>
<td>6 (9.4)</td>
<td>0 (0.0)</td>
<td>0.08</td>
</tr>
<tr>
<td>Long disease, n (%)</td>
<td>12 (18.8)</td>
<td>9 (30.0)</td>
<td>0.80</td>
</tr>
<tr>
<td>Tuberculosis, n (%)</td>
<td>1 (1.6)</td>
<td>3 (10.0)</td>
<td>0.00</td>
</tr>
<tr>
<td>Asthma, n (%)</td>
<td>1 (1.6)</td>
<td>3 (10.0)</td>
<td>1.00</td>
</tr>
<tr>
<td>Contracted IL1 n (%)</td>
<td>14 (22.0)</td>
<td>15 (50.0)</td>
<td>0.15</td>
</tr>
<tr>
<td>Pneumococcal conjugate vaccine, n (%)</td>
<td>11 (17.2)</td>
<td>4 (13.3)</td>
<td>0.25</td>
</tr>
<tr>
<td>DTP:IPV:Si-Hb vaccine 1 dose, n (%)</td>
<td>24 (37.5)</td>
<td>22 (73.3)</td>
<td>0.45</td>
</tr>
<tr>
<td>DTP:IPV:Si-Hb vaccine 3 doses, n (%)</td>
<td>28 (44.4)</td>
<td>18 (60.0)</td>
<td>0.24</td>
</tr>
<tr>
<td>Influenza A/H1N1 vaccine</td>
<td>4 (6.3)</td>
<td>8 (26.7)</td>
<td>0.06</td>
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</table>

**Hospitalization follow-up**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SIRS-SHOCK PNEUMONIA n=64</th>
<th>SEVERE PNEUMONIA n=30</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pneumonia complication, n (%)</td>
<td>5 (7.8)</td>
<td>14 (46.7)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Length of hospitalization, days, median (Q1-Q3)</td>
<td>3 (2.3)</td>
<td>7 (8.8–12.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Death, n (%)</td>
<td>0</td>
<td>0 (0.0)</td>
<td>0.16</td>
</tr>
</tbody>
</table>

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, transcripomics and omics analyses provided new data on the inflammatory pathways that are activated during mixed infection and related to synergistic induction of pro-inflammatory chemokine IP-10 in mixed infected cells. Our observations are consistent with a recent study describing IP-10 induction as host-proteins signature of both viral and bacterial infections28. Of the differentially-expressed genes observed in mixed infected MDMs, the transcription factors STAT-1 and IFN-γ 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 infections upregulate miR-155 in macrophages and dendritic cells and also regulate expression of cytokine signaling 1 (SOCS1), suggesting the existence of a miRNA/JAK-STAT/SOCS regulatory pathway during viral infection29. Our study suggests co-infection leads to overexpression of miR-200a-3p, which in turn targets and downregulates the JAK-STAT regulator SOCS1 and consequently increases CXCL10 (IP-10) expression. Interestingly, a complementary in-silico approach revealed that several microRNAs that were found dysregulated in our experiments of 1AV and 1PV co-infection of MDMs or HAE3C, might target several genes
Table 2. Only CIP levels are expressed in mg L−1 otherwise in μg ml−1. Values are expressed as median (IQR) in pg ml−1. Differences between groups were compared using unpaired Mann-Whitney test significant changes (P<0.05) are in bold.

<table>
<thead>
<tr>
<th>Blood-based Markers</th>
<th>NON-SEVERE PNEUMONIA n = 44</th>
<th>SEVERE PNEUMONIA n = 39</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3 (mg/L)</td>
<td>2.9 (1.4–6.0)</td>
<td>162.0 (26–157)</td>
<td>0.01</td>
</tr>
<tr>
<td>CRP (mg/L)</td>
<td>0.1 (0.1–0.3)</td>
<td>1.14 (0.5–2.7)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ALT (IU/L)</td>
<td>150.0 (72.6–324)</td>
<td>277.6 (155.9–657.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>AST (IU/L)</td>
<td>103 (3–20.6)</td>
<td>17.0 (8.3–31.3)</td>
<td>0.07</td>
</tr>
<tr>
<td>AST/ALT ratio</td>
<td>1.3 (0.8–2.5)</td>
<td>4.4 (1.1–8.9)</td>
<td>0.18</td>
</tr>
<tr>
<td>IL-10 (pg/ml)</td>
<td>671 (156–1,684)</td>
<td>486 (165–1,280)</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-6 (pg/ml)</td>
<td>164 (61.1–379.5)</td>
<td>358 (159–1,279)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>IL-8 (pg/ml)</td>
<td>53 (11–144.6)</td>
<td>117.2 (28–303.5)</td>
<td>0.03</td>
</tr>
<tr>
<td>IL-9 (pg/ml)</td>
<td>51.4 (15.5–92.3)</td>
<td>68.8 (24–117.7)</td>
<td>0.37</td>
</tr>
<tr>
<td>MCP-1 (pg/ml)</td>
<td>26 (0–0.35)</td>
<td>108 (0.1–0.36)</td>
<td>0.19</td>
</tr>
<tr>
<td>MIP-10 (pg/ml)</td>
<td>1,696 (127.6–2,315.1)</td>
<td>260 (1,305–3,813)</td>
<td>0.81</td>
</tr>
<tr>
<td>PDCD3-B (pg/ml)</td>
<td>6,773 (6,242–28,389)</td>
<td>14,111 (7,055–36,160)</td>
<td>0.29</td>
</tr>
<tr>
<td>RANTES (pg/ml)</td>
<td>34,386 (24,276–48,984)</td>
<td>86,318 (23,565–153,870)</td>
<td>0.55</td>
</tr>
<tr>
<td>VEGF (pg/ml)</td>
<td>1,781 (577.4–4,035)</td>
<td>3,180 (1,405–5,915)</td>
<td>0.11</td>
</tr>
</tbody>
</table>

of SOCS3 family and play similar role than miR 200a 3p. Indeed, miRNA 142 3p might target SOCS3 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 signalling 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 67% of cases: 51.3% of which were viral, viral-bacterial or bacterial-viral co-detected only in nasal aspirates, 31.8% of which co-detected in both nasal aspirates and blood samples. These data are consistent with previous etiologic studies of pediatric CAP (57).

5. pneumoniae was the major bacteria identified in blood (19/74; 25.7%) and mainly co-detected with respiratory viruses in 76% (16/18; 94.4%). We observed a very high diversity of viral and bacterial associations in biological samples from children with pneumonia. In comparison with IAV and EP14 combination evaluated in vitro, no pneumonia cases were singly influenza and pneumococcal 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 subtype 14 using our molecular typing test). IVA and SP14 combination seems to be the narrowest pathogen co-detection to that in vitro investigated. Clinically, this co-detection was associated with a very high IP-10 expression and a very severe pneumococcal 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 pathogens 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 capillaries (72) and were associated with high mortality rate, especially among patients with CAP (57). Additionally, the IP-10-CXCR3 axis has been related to acute immune lung injury and lymphocytes apoptosis during the development of severe acute respiratory syndrome (SARS) (56). Moreover, an in vitro 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 pneumococci, severe immunopathology and mortality (56). In this study, markedly elevated IP-10 (928 pg ml−1) combined with the highest CPT level (74 μg ml−1) were quantitated in the serum sample of a child who died, in whom a pneumonia (severe type V) was identified in the blood (5C3 and blood culture) and co-detected with Staphylococcus pneumoniae type 8 in nasal aspirate. These observations suggest an interrelationship between co-detections, 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 HIV and H1N1) 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
suggest that mixed respiratory infections and IFN-10 may play major, interconnected roles in the pathogenesis of pneumonia. Clinically, assessment and monitoring of induced IFN-10 serum 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 (A/Va/Moscow/30/09) was obtained from the National Influenza Reference Center (Lyon, France). Influenza B: Influenza A: Sp. Streptococcus pneumoniae, serotype 6 AB, 6A, 3, 4, 5, 6, 7, 8, 0, 23, E 17, 35, 14, 39, 19 E 14, 7, 16 E 19 A; NT: non-typhoidal; S. typhimurium: S. typhimurium: Hpa: Haemophilus influenzae type b.

Cell culture conditions. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of a control donors (male/female ratio 1/3) range of age: 21 - 65 years using Ficol-Hype Prep (GE Healthcare Life Sciences, Little Chalfont, United Kingdom) and monocytes were purified using magnetic C314 MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany). The purity of isolated C314+ 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 glutamine supplemented with 10% heat-inactivated FBS and
10 ng ml⁻¹ M-CSF (Miltenyi Biotec). THP-1 MDMs were obtained by culturing cells with 10 ng ml⁻¹ phorbol myristate acetate (PMA; Invitrogen, Thermo, France) for 72 hours. Human airway epithelial cells (HAE, bronchial cell type) originated from a 54 years old woman with no pathology reported (batch number MD6650) were provided by Mucilair (Eppihal, Geneva, Switzerland). Staining, tissue integrity (TEER), mucus production and ciliating 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 (H1N1 hemaglutinin gene, provided by Dr AD Osterhaus from the Department of Virology, Erasmus Medical Center, 3015 CE Rotterdam, Netherlands) and SP (autolysin A gene, LytA, unpublished home-made primers and probe design) in MDMs. The primers and probes ( primer F 5′-GAAATGCCCCATCAATCTGGGTA-3′, primer R 5′-GTAATTCCGAACCTTGGCAGTTC-3′, probe 5′-FAM-TAGGGTCTGCTGAAGGACTAAC-TAMRA-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 92°C for 30 seconds and 55°C with fluorescent detection (annealing and extension). Absolute quantification of viral hemaglutinin and pneumococcal LytA copy number were calculated by 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, permeabilised in PBS 0.1% Triton X-110, and incubated with PBS containing 1% BSA and 0.1% human serum to block non-specific binding. Mouse monoclonal anti-Influenza A Virus Nucleoprotein (Ab99211; Abcam, Cambridge, United Kingdom) and goat anti-mouse IgG H&L (AF488; A11001; Life Technologies) were used to detect IAV. Rabbit polyclonal anti-Streptococcus pneumoniae antibody (Ab10428; Abcam) and goat anti-rabbit IgG H&L (AF547; A10080; Abcam) were used to detect SP. Cells were imaged using a Leica DMI 300B microscope equipped for fluorescence expression imaging.

Gene expression profiling. Total cellular mRNA was purified using the XNeasy kit (Qiagen, Hilden, Germany). Reverse transcription of total mRNA was performed using the RT First Strand Kit (DAgBiociences, Hilden, Germany). The expression of 64 genes involved in the human innate and adaptive immune responses was evaluated using the RT profiler™ PNR Array (DAgBiociences) according to the manufacturer’s recommendations. The ΔΔCt method was applied to calculate the fold changes in gene expression for each gene relative to uninfected control cells using the web-based RT profiler PNR Array Data Analysis software (DAgBiociences).

MicroRNA profiling array. Total cellular microRNAs were purified using the mirNeary Mini kit (Qiagen) and reverse transcribed using the miScript Reverse Transcription kit (Qiagen). The profiling of 84 microRNAs was performed using the human immunopathology microRNA PCR Array Kit (Qiagen) according to the manufacturer’s instructions. Data were analyzed using the miScript microRNA PCR array data analysis web portal.

In silico miRNA target prediction. miRNA target genes were retrieved and compiled using TargetScan® and microRNA.org resource. The interactions between miRNAs and intracellular pathways were predicted using DANA-miPath v2.4.

Monocyte-derived macrophage (MDM) co-infection model. Human primary CD14+ or THP-1 MDMs were seeded in 24-well plates (0.5 × 10⁶ 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 24 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⁻¹) 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 viabilities were determined by flow-cytometry using the FITC Annexin V apoptosis detection kit (BD Biosciences), according to the manufacturer’s instructions.

Functional analysis of miR-203a-3p. For miR-203a-3p inhibition/mimic assays, 1 × 10⁶ THP-1 MDMs were transfected with either 30 nM miR Non A (Bionova, Singapore) or 1 × 10⁶ MDMs with 30 nM miR-203a-3p mimic (Bionova, Singapore). mRNA inhibition/ Negative Control #1 (Bionova, Singapore) or miR Non A, Negative Control #1 (Bionova, Singapore), all Life Technologies in OptiMEM Life Technologies using Lipofectamine®2000 for 12 well plates. After 18 hours, cells were washed and infected as previously described. The efficiency of the miR-203a-3p inhibition/mimic was evaluated 24 hours after infection by purifying total mRNA (miRNA A) using miRNA Isolation Kit A, Ambion, Life Technologies), reverse transcription and specific amplification of miR-203a-3p using the TaqMan MicroRNA Reverse Transcription kit (Life Technologies, #446696) combined with a specific miRNA probe assay (Life Technologies, #442792). In this assay, fold change have been defined by the ΔΔCt method using control RNA-48 as reference miRNA. Total miRNA was purified from transfected and infected MDMs using the XNeasy kit (Qiagen) and primers used to amplify transforming growth factor beta-2 (TGFB2) probe (F: 5′-GATCTGGGCCCCATCCTTGAC-3′, R: 5′-GCCCAACGGAGCAGG-3′, probes 5′-GCCCAACGGAGCAGG-3′, 5′-GCCCAACGGAGCAGG-3′, and β-Actin (F: 5′-GCGATCTGGGCCCCATCCTTGAC-3′, R: 5′-GCCCAACGGAGCAGG-3′, Rabbit...
<table>
<thead>
<tr>
<th>NON-SEVERE PNEUMONIA (n = 44)</th>
<th>SEVERE PNEUMONIA (n = 30)</th>
<th>TOTAL (n = 74)</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>NOS defense</td>
<td>PB-3</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>Single detection</td>
<td>PB-3</td>
<td>2 (4.2)</td>
</tr>
<tr>
<td>Multiple detection of pathogens</td>
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<td>2 (4.2)</td>
</tr>
<tr>
<td>TOTAL</td>
<td>PB-3</td>
<td>2 (4.2)</td>
</tr>
</tbody>
</table>

Values are expressed in pg/ml. IP-10 concentrations differences between groups were compared using unpaired Mann-Whitney test; significant changes (P < 0.05) are in bold.

![Diagram of human macrophage-derived NDMs and IP-10 signaling pathway](image)

**Figure 4.** 1AV and SD co-infection of MDMs exacerbates induction of IP-10. Putative role for miR-203a-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-203a-3p was synergistically induced following mixed 1AV/SP infection of human MDMs and found to indirectly regulate JAK/STAT10/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 infection (F = 0.03) and non-severe pneumonia (P < 0.001).

Polyclonal antibodies against 3-actin (#ab8227), SOCS3 (#ab13930), SOCS5 (#ab16030) and goat polyclonal anti-influenza A/H1N1 (Ibali 9979, all Abscan) were used for Western blotting.

**Multiplex immunoassay of inflammatory mediators.** Serum samples and cell culture supernatants were screened for the presence of 57 human cytokines and chemokines using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Bio-Rad, Hercules, California, USA) on a FLEXMAP 3D 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 2 year-old (study protocol published in), a total of 74 patients (44 male, 30 female) admitted to the Hospital Pediatrico Niños de Acosta Sal, 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, ≥60/min; age 2-11 months, ≥50/min; age 1-5 years, ≥40/min), and 3) absence of wheezing at auscultation, and, 4) first symptoms appearing within the last 24 hours, and 5) radiological confirmation of pneumonia as per WHO guidelines. Based on these primary criteria, a final pediatric pneumonia diagnosis was established. For patients with this final diagnosis, the following clinical signs were assessed: conjunctival injection, cough, tachypnea, tachycardia, fever, vomiting, and increased work of breathing. Additional signs were identified as follows: coughing, wheezing, stridor, and cyanosis. The presence of these signs was assessed by the primary care team at the time of admission. The study was approved by the Institutional Review Board of the Hospital Pediatrico Niños de Acosta Sal, San Lorenzo, Paraguay.
Clinical and molecular analysis. Nasopharyngeal aspirates (NAAs) 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 Saphyrophilus aurus. Streptococcus pneumoniae and Haemophilus influenzae type B. S. pneumoniae serotypes were defined using real-time PCR assay targeting the 40 most frequently represented serotypes or serogroups according to protocol developed by Messaoudi et al. 16. Serum C-reactive protein (CRP), Assayfit, S., Charleston, Missouri, United States) and Pneumococcal (PCR, VDAS L.A.R., M., M.B, Inc)) were quantified from whole blood samples. Multiplex real-time non quantitative PCR (Fast-Track Diagnostic, Siemens, Malaga) was used to detect 19 viruses and five bacteria in respiratory specimens (NAAs and pharyngeal exudates). Multiplex detection was defined as 1) PCR-positive for multiple viruses in NAAs, 2) positive blood culture or PCR-positive for multiple bacteria in blood or 2) PCR-positive for one or multiple viruses in NAAs and one or multiple bacteria in blood (identified by PCR and blood culture).

Ethical approval. The study protocol, informed consent statement, clinical research forms, any amendments and all other study documents were submitted to and approved by the Ethical Committee of the Institute of Investigation on Children's Health, the Universidad Nacional de Asunción (UNAS-UNA) and the Hospital Pediatrico Ninos de Acaosta, Asunción, Paraguay. 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 unequal variances t-tests. Comparative analyses between experimental conditions (i.e., MOCK, IAV, SP or IAV + SP) were performed with one-way ANOVA with Tukey's post hoc test or Kruskal Wallis analysis with Dunn's post hoc tests. Receiver operating curves (ROC) analysis was used to determine the optimal cut-off thresholds for 1P0 to differentiate between non-severe and severe pneumococcal cases. P < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism (La Jolla, California, United States).

References


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Author Contributions


Additional information

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Article 2. RSV infection in macrophages promotes IP-10 expression during bacterial co-infection
RSV infection in human macrophages promotes CXCL10/IP-10 expression during bacterial co-infection

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

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

1. Introduction

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

Respiratory syncytial virus (RSV) is the most important cause of death in very young children, and global RSV disease burden is estimated in approximately 200 000 deaths and over three million
hospitalizations per year. Currently, the most common causative agent of viral LRI is RSV [11, 12]. In contrast with Influenza viruses, prophylactic and therapeutic approaches for RSV remain very limited and new vaccine and antiviral strategies are urgently required. Among bacterial pathogens, Streptococcus pneumoniae (Sp) is the most common cause of severe pneumonia. It is a commensal of the nasopharynx and most carriage episodes do not result in disease [13] but pneumococcal pneumonia results in approximately half a million deaths in children younger than 5 years each year worldwide.

A large retrospective study performed recently provided evidence for an interaction between RSV and pneumococcal pneumonia [14]. In children with acute LRI, RSV was associated with increased colonization by Sp, in a similar way to that was described in the context of influenza infection [15-19], and although we do not know the exact mechanisms of this interaction, it is assumed that it is due to the increase of attachment sites to the bacteria in the epithelial cells caused by viruses. The prevalence of RSV/Sp co-infection varies in approximately 40% of samples from pediatric RSV infections [20-22] and in vitro and in vivo studies show significant increase in disease severity in this context [15, 23-25].

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

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

2. Results and Discussion

First, monocytes were isolated from human peripheral blood mononuclear cells (PBMC) and differentiated into macrophages as previously described [1]. These human MDMs were then exposed to RSV (Long strain, ATCC VR-26) at different multiplicity of infection (MOI) to investigate the kinetics of infection by monitoring of genome copy number using a specific RT-qPCR on cell lysates harvested at different time-points (Figure 1A) As a control, immortalized monocyte-like THP-1 cells were infected in parallel with the same protocol. Our results confirmed that MDMs can be effectively infected by RSV, similarly to THP-1, but that viral replication remains very limited in the case of MDMs. These observations were in good agreement with early works that showed that RSV replication in macrophages was possible but with a limited extent [35-36]. Indeed, we observed a similar kinetic profile whatever the MOI used, with a replication peak at 8hpi, followed by a stabilization or a decrease of genome copy number at 8hpi for THP-1 and MDMs, respectively (Figure 1A). In contrast to that is usually described in cell lines (e.g. Hep-2 or A549), we did not observe marked cytopathic effects in infected MDMs, but rather a slightly higher number of dying cells (data not shown). To complete these observations, we then monitored cell death during RSV
infection using flow cytometry (Annexin V/propidium iodide staining) (Figure 1B). Our results confirmed that RSV infection increased cell death in MDMs, with a significant decrease of survival at a MOI of 10 at 48 hpi (Figure 1B). In parallel, we performed immunofluorescence microscopy of RSV-infected MDMs, using a specific antibody to detect RSV F protein (ab20450; Abcam, Cambridge, UK) (Figure 1C). At a MOI of 1 at 8 hpi, around 10–15% of cell monolayer was positive for RSV antigen staining (Figure 1C), indicating an efficient infection, and possibly an initial limited viral replication, as RSV F staining still progressively increases from early stages to 8 hpi (data not shown). Interestingly, the level of apoptosis in RSV-infected cells (Figure 1B) is not completely correlated with the number of infected cells (Figure 1C), suggesting that apoptosis is also induced in non-infected cells. Altogether, these results indicate that human MDMs are susceptible to RSV infection. Moreover, viral replication occurs, to a limited extent, in slight disagreement with previous observations indicating abortive infection in lung macrophages [37–39].

**Figure 1.** Characterization of RSV infection in human monocyte-derived macrophages (MDMs).

Monocytes were isolated from human peripheral blood mononuclear cells (PBMC) and differentiated into macrophages as previously described [1]. RSV (Lew strain, ATCC VR-26) was propagated in HEP-2 cells and isolated as described previously [43]. A. THP-1 or MDMs cells were exposed to RSV at different multiplicities of infection (MOI; 0.1, 1 and 10) to investigate the kinetics of infection over time.
Uninfected control explants were exposed to cell culture medium. At different time-points, total cell extracts were harvested and analyzed by multiplex real-time RT-qPCR to quantify RSV F fusion gene. The RT-qPCR reaction was performed using the AgPath-ID™ One-Step RT-PCR kit (Life Technologies; Carlsbad, California, USA) according to the manufacturer’s instructions. Samples with a cycle threshold (Ct) value of ≥30 were recorded as negative. A standard curve was prepared using serially diluted RNA extracts from a known quantity and used to calculate genomic copies/mL. In the same experimental condition, cell survival in infected MDMs was monitored using flow cytometry, using the FITC/Annexin V apoptosis detection kit (BD Biosciences), according to the manufacturer’s instructions. Percent survival is expressed compared to viable cells measured at T=0hpi. Immunofluorescence microscopy of MDMs infected by RSV (MOI of 1) at 8hpi immunofluorescence protocol was performed as previously published ([4]). RSV F antigen (Green), Nuclei stained by DAPI (Blue). Each experiment was performed in duplicate, for two separate experiments.

In a second step, we used this MDM model to study RSV/Sp mixed infection. We have previously demonstrated that human MDMs were permissive to Sp infection ([1]). Using a similar experimental approach, MDMs were first infected by RSV at a MOI of 1 and then mock-infected or infected by Sp at 1 CFU/cell, 4h after viral infection. Viral kinetics were monitored by RT-qPCR for 48 hpi (Figure 2A) and by measure of RSV viral load (TCID50/mL) in the supernatants (Figure 2B). Whereas the genome copy number remained unaffected by the subsequent challenge with Sp, we observed a significant decrease of viral production in the context of Sp co-infection, notably at 8hpi, with more than three log10 difference of TCID50/mL in comparison with single RSV infection (Figure 2B).
Figure 5. Characterization of RSV/9p mixed infection in human monocyte-derived macrophages (MDMs). Human MDMs were infected by RSV at a MOI of 1. After 4h of viral infection, cells were mock-infected or infected by 9p at 1 CFU/cell. Encapsulated Streptococcus pneumoniae serotype 14 was obtained from the National Reference Center for Streptococci (Dept of Medical Microbiology, Aachen, Germany). Pneumococci were opsonized in anti-pneumococcal immune serum and incubated for 30 min at 37°C prior to infections for all experiments. MDM cells showed significant loss of viability following overnight incubation with S. pneumoniae, and therefore gentamicin (15μg/ml) was added to all wells at the 24h time point to prevent bacterial overgrowth and loss of cell viability. A. At different time-points, total cell extracts were harvested and analysed by multiple real-time RT-qPCR to quantify RSV. B. RSV viral production in supernatants was performed by quantitative viral titers (TCID50/mL) with a limit-dilution assay and using the Reed & Muench statistical method. Results are expressed as log10 TCID50/mL. C. In the same experimental condition, cell survival in infected MDMs was monitored using flow cytometry, with the ITTC/Anexin V apoptosis detection kit (BD Biosciences), according to the manufacturer's instructions. Percent survival is expressed compared to viable cells measured at T=0 hpi. D. E. Co-infection alters the timing and extent of IFN-10 expression in human monocyte-derived macrophages (MDMs). Human MDMs were infected by RSV at a MOI of 1. After 4h of viral infection, cells were mock-infected or infected by 9p at 1 CFU/cell. At different time-points, total cell extracts were harvested and analysed by multiple real-time RT-qPCR to quantify CXCL10 mRNA levels. Total mRNA was purified from transfected and infected MDMs using the RNeasy kit (Qagen) and specific primers were used to amplify CXCL10 (F: 5'-GGGATTCAACAGTACTTC-3', R: 5'-TGAATGGCATCTTGTATTTT-3') and β-Actin for normalization (F: 5'-CTCTTGACGCCCTTTTCC-3', R: 5'-GACACGTGTTGGCCTACG-3'). E. Similar strategy with
different combinations of RSV and Sp, at 8hpi. Each experiment was performed in duplicate, for two separate experiments.

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

RSV infection is associated with increased expression of various cytokines [29]. After characterizing the simple and mixed infections in macrophages, innate immune response to single or mixed RSV infection in MDM cells was examined by measuring changes in the expression of the CXCL10 gene. The mRNA levels of CXCL10 were measured in the cells culture of mock, single or mixed infections at different time points after infection (Figure 2D). In our experimental conditions, Sp single infection did not induce the expression of IFN-γ, in contrast with our previously published study [1], most probably due to differences of protocols, which were adopted for RSV infection (e.g. lower cell confluence). In the context of single viral infection, we observed a dramatic increase of IFN-10 expression at 15hpi, with a fold change of more than 14,000 compared to mock (Figure 2D). This high value could be explained by the relative low level of IFN-10 expressed in mock (CT value = 40) compared to infection (CT value = 27). Interestingly, this increase of CXCL10 expression was observed in the context of a very limited viral replication at the same time-point (Figure 2D and Figure 2B). A similar increase of CXCL10 expression was observed in the context of RSV/Sp mixed infection, but much earlier, at 8hpi (Figure 2D). This observation suggests co-infection induces an innate immune response more rapidly. To further investigate the possible synergy that could occur in term of activation of IFN-10 expression in response to mixed infections, we infected MDMs with 2 different MOIs of RSV (MOI 1 and 10) together with a single MOI of Sp (0.1, 1 and 10) CFU/cell) and monitored CXCL10 expression by RT-qPCR (Figure 2E). As expected, IFN-10 expression was stimulated with increasing quantity of viral inoculum. At a MOI of 1, CXCL10 expression was strongly induced in presence of Sp (Figure 2E). Interestingly, at a MOI of 10, we observed a stronger increase of CXCL10 expression in the context of Sp co-infection (Figure 2E). Altogether, these results suggest that Sp could contribute to a faster and possibly higher level of CXCL10 expression induced by RSV infection in human MDMs.

3. Conclusion and Perspectives

In conclusion, we have shown that human MDMs constitute an interesting and biologically relevant experimental model to study RSV/Sp co-infections. Our data support that early stages of RSV infection strongly trigger the innate immune response in human macrophages cells, in good agreement with previously published studies [29, 40]. For example, Levitz and colleagues have recently described a strong induction of IL-6 and CXCL5 in MDMs infected by different clinical isolates of RSV [40]. In a mice model, CXCL10 was demonstrated to have an important protective role to the host by reducing viral load and pathogenesis [41]. In the context of mixed infection, we have shown that expression of CXCL10 occurred earlier than in single RSV infection (16h delay, Figure 2E), suggesting a faster and possibly stronger response of macrophages, also with good agreement with other in vitro and in vivo studies [42].

We have shown that CXCL10 expression was strongly increased during infection, even if the level of viral replication appears very limited in MDMs. Based on this, we can speculate that strategies to control the innate response could constitute an interesting option in the treatment of patients with RSV infection. In this study, although RSV infection strongly mitigated by macrophages after 24hpi, we have shown that bacterial co-infection contributes to alter the timing and extent of innate immune response, as illustrated by CXCL10 expression. We previously obtained
quite similar results in a model of influenza/S. pneumoniae co-infection infection in human MDMs, suggesting that common underlying mechanisms in different context of viral infection [4]. Future investigations may lead to the identification of key cell signaling pathways that mediate the host antiviral response to infection, which in turn could lead to the identification of novel drug targeting pro-inflammatory responses during infection by RSV. With increasing evidence of interactions between RSV and bacteria in the respiratory tract, it is important to better understand interconnections between pathogens and their combined effect on disease severity to develop future strategies for the prevention and treatment of RSV-induced severe acute LRIs.

4. Materials and Methods

**Cell lines and culture conditions.** Hep-2 cells (ATCC CCL-23) were maintained in Eagle’s minimal essential medium (EMEM) with 2 mM L-glutamine and 10% FBS and maintained at 37 °C, 5% CO2. THP-1 MDMs were grown in RPMI 1640 medium L-glutamine containing 10% FBS and maintained at 37 °C, 5% CO2. THP-1 cells (ATCC TIB-202) were induced to differentiate into macrophages by incubation with 10 ng/mL phorbol myristate acetate (PMA, Invitrogen, Toulouse, France) for 72 hours.

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

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

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

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

**IP-10 mRNA expression.** For real-time quantitative PCR, total RNA was extracted using the RNeasy Mini Kit (Qiagen). Reverse-transcription was performed on 1 µg of total RNAs using the SuperScript II enzyme (Invitrogen) at 42°C. Quantification of IP-10 mRNA levels was performed by real-time quantitative PCR using specific primers for IP-10: (F: 5'
-CTGGGATTCAAGGAGTACCTC-3', R: 5' TGGGGCCCTGATTTGAGAT-3') and β-ACTIN (F: 5'
-CTCTCCAGGCTTCTCCCT-3', R: 5' AGACATGTGGTGAGCTACAG-3') for normalization.

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

Immunofluorescence Microscopy. Infected cells were washed with PBS, fixed with 4%
paraformaldehyde for 20 minutes at room temperature, permeabilized with PBS-0.1% Triton X-100,
and incubated with PBS containing 1% BSA and 3% human serum to block non-specific binding.
Reagents used for immunofluorescence in this study were goat anti-RSV (ab20745; Abcam,
Cambridge, UK). Antibody incubations were performed for 2 hours, followed by three washes with
PBS. Bound RSV primary antibodies were detected using FITC conjugated donkey anti-goat
antibody (ab6811; Abcam, Cambridge, UK). Cells were imaged using a Leica DMI 3000B microscope
equipped for fluorescence imaging. Images were rendered by ImageJ Software (NIH, Maryland,
United States).

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

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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.

Ethical statement: Blood donation was obtained from healthy adult volunteers (Etablissement Français du
Sang, Lyon Garibaldi, France). Written informed consent from each subject were obtained (national procedure
used for blood donation).

Abbreviations

RSV Respiratory syncytial virus
S. pneumoniae Streptococcus pneumoniae
MDMs Human monocyte-derived macrophages
LRIs Lower respiratory infections
IP-10 Interferon-γ-inducing protein 10

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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-kB. MAPK are involved in RSV replication in human airway epithelial cells [488]. The precise trigger of NF-kB activation is uncertain and may involve oxidative stress, virus replication, viral nucleic acid, or expression of individual viral proteins. NF-kB 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
Role of p53/NF-κB functional balance in RSV-induced inflammation and immune responses

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

Short title
Role of p53/NF-κB balance in RSV infection
ABSTRACT

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

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

Our current knowledge of the cellular biology of RSV has been mainly obtained through studies focused on the immune and inflammatory responses. RSV infection was shown to upregulate the expression of host genes involved in the antiviral and cell-mediated immune responses, such as genes coding for interferons (IFN-alpha/beta) or several cytokines/chemokines [12–14]. Interestingly, while the nuclear factor-kappaB (NF-kB)/REI family of transcription factors plays a central role in coordinating the expression of a wide variety of genes that control immune responses [15], several studies have highlighted the multiple levels of interplay between the NF-kB pathway and RSV during the time course of infection [16–22]. In that regard, viral non-structural proteins NS1 and NS2 have been shown to indirectly contribute to the RSV-induced activation of NF-kB [23–34].

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

In this study, we confirmed that RSV strongly impairs p53 transcriptional activity at late stages of the infection cycle, notably via a proteasome-dependent degradation. We also demonstrate that NS1 and NS2 contribute to the abrogation of p53 activity, and used different experimental strategies (e.g. siRNA, small molecules) to underlie the antiviral contribution of p53 in the context of RSV infection.

We finally propose a model by which RSV infection strongly induces a disequilibrium of the p53/NF-kB functional balance, which probably contributes to the up-regulation of several proinflammatory cytokines and chemokines.
RESULTS

RSV impairs p53 transcriptional activity at late stages of infection.

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

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

We then investigated the impact of RSV infection on p53 stability. We mock-infected or infected A549 cells at MOI of 1 for 24 or 48 h, and then analyzed p53 stability by monitoring protein levels over a 50-min period, after treatment with 50 μM of cycloheximide (CHX) protein synthesis inhibitor (Fig. 2A). As expected, the estimated p53 half-life in mock was 30 min, in agreement with previous works using a similar cellular model and experimental conditions [29]. In contrast, we observed a faster decrease of p53 RPL in the context of RSV infection, with an estimated half-life below 10 min (Fig. 2A). Interestingly, after 10 min of CHX treatment at 48 hpi, p53 levels were undetectable by western blot (Fig. 2A). We then further investigated this strong destabilization of p53 at 48 hpi in presence of proteasome inhibitor MG132 and then observed that the decrease of p53 RPL was blocked.
in this condition (Fig. 2B). Altogether, these results suggest that RSV infection decreases p53 stability via a proteasome-dependent pathway.

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

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

To further investigate the role of p53 in RSV infection, we first evaluated the impact of endogenous p53 silencing on viral production. A549 cells were transfected with either a non-specific siRNA (si-Ctrl) or a pool of siRNAs targeting p53. Forty-eight hours post-transfection, cells were infected with
RSV at a MOI of 1 and viral supernatants and cell extracts were harvested at 48 hpi. As expected, western blots confirmed that p33 levels were reduced in cells transfected by si-p33 compared to si-Ctrl (Fig. 4A). We also observed that RSV genome copy numbers were significantly increased (2-fold increase, \( P < 0.001 \)) in supernatants of si-p33 transfected cells, compared to those of si- Ctrl transfected cells. This increase was confirmed by infectious titers (Fig. 4A). To complete our observations, we followed a transient expression approach in H1299 human lung cells (Fig. 4B), which constitutively lack the expression of full-length p33. H1299 cells were transfected with either an empty plasmid or a plasmid expressing p33. Forty-eight hours after transfection, cells were infected with RSV (MOI of 1) and the impact of transient expression on viral production was assessed at 48 hpi. The adequate transient expression of p33 was confirmed by western blot. In these conditions, viral production was significantly lower in cells transfected with p33, with more than 2.5-fold decrease on genome copy numbers (\( P < 0.001 \)), and a 2 log10 reduction on infectious viral titers (Fig. 4B). In parallel, we advantageously used Nutilin-3, a small molecule known to bind the Mdm2 E3-ubiquitin ligase, therefore causing the stabilization and activation of endogenous p33 [31]. A549 cells were mock infected or infected with RSV (MOI of 1), in the presence of either Nutilin-3 (10 \( \mu \)M) or control DMSO (Ctrl). Treatment with Nutilin-3 significantly decreased viral production compared to control, with almost 3-fold decrease on genome copy number (\( P < 0.001 \)) and a 3-log10 reduction on viral titers (Fig. 4C). These results were consistent with our observations using siRNA and transient expression, suggesting an antiviral contribution of p33 in the context of RSV infection.

RSV infection modulates the p33/NF-κB balance via a NS2-PI3K/Akt pathway. The p33 and NF-κB pathways are known to be interconnected and responsible for reciprocal functional balance at the crossroads of several cellular processes [32]. Based on previously described data on the interplay between RSV and NF-κB [16–22], we mock infected or infected A549 cells in the conditions that lead to a marked decrease of p33 activity (MOI 1, 48 hpi, as described above), and then used a fluorescent reporter assay to quantify the phosphorylation of p65, as a surrogate of NF-κB activity (Fig. 5A). In these conditions, we observed a strong and significant increase on the relative fluorescence intensity (96-fold increase, \( P < 0.05 \)) (Fig. 5A), indicating that the RSV-induced decrease of p33 activity is inversely correlated with NF-κB activity. To complete this observation, we also performed similar experiment in the context of Nutilin-3 or DMSO (Ctrl) treatment. The stabilization and activation of p33 by Nutilin-3 in RSV-infected cells was associated with a significant reduction on fluorescence intensity compared to control (3.3-fold decrease, \( P < 0.001 \), Fig. 5A).

Additionally, we also monitored p65 phosphorylation in conditions that NS1 or NS2 transient expression (2 \( \mu \)g of plasmid, 48 h post transfection) inhibits p33 transcriptional activity (Fig. 5B). We observed that both NS1 and NS2 induce a strong increase of p65 phosphorylation, reflecting an upregulation of NF-κB activity, in agreement with data published by Biko and colleagues [23].
Altogether, these observations suggest that the NS1/NS2-induced modulation of p53 and NF-κB would be interconnected and inversely correlated.

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

Finally, NF-κB has been extensively documented for its regulatory role on a large panel of cytokine/chemokine expression. As our results indicate a likely modulation of the p53/NF-κB balance in the context of RSV infection, we investigated the contribution of p53 activity to the regulation of cytokines and chemokines expression during infection. To that end, A549 cells were pretreated with either a si-Ctrl or si-p53 for 48 h and then infected with RSV for 48 h (Fig. 5E). A panel of 22 different cytokines and chemokines were quantified in supernatants by using antibody-based assay (Bio-Plex Pro, BioRad). Results are represented as a heatmap showing for each condition the fold change compared to the mock (si-Ctrl/si-p53 – Fig. 5E). While a large number of cytokines such as G-CSF, IL-1α or IL-6 appeared unaffected by the silencing of endogenous p53 expression (Fig. 5E), others were significantly deregulated, as IL-9, IL-10, VEGF and notably RANTES (Fig. 5E).

Altogether, our results suggest that in the context of RSV infection, p53 could play a role as a regulator of cytokines and chemokines expression, that NS2 would inhibit in a PI3/Akt-dependent manner.
DISCUSSION

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

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

Importantly, our work also highlights the contribution of the two non-structural proteins NS1 and NS2 to the inhibition of p53 transcriptional activity at post-translational level (Fig. 3). While the mechanism of NS1-induced inhibition remains to be deciphered, our results suggest that, in the case of NS2, this regulation occurs through the activation of Akt, hence promoting the degradation of p53 (Fig. 3 and Fig. 5D, and proposed model Fig. 5F). We expect future work investigating the possible direct interaction between NS1 and p53 could better explain our observations. In the meantime, NS1 and NS2 are known to play important roles in the modulation of cellular responses like the IFN response, and more largely in RSV/host interactions [12–14, 30]. Interestingly, two studies have also reported the possible contribution of other RSV proteins (Matrix and Fusion proteins) to the regulation of p53-
dependent cellular processes [27, 28]. Altogether, these observations clearly reinforce the idea that p53 constitutes a key factor in the host response to RSV infection, for which it would be a privileged target of RSV.

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

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

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

Cell lines and viral strain. Human lung epithelial A549 cells (ATCC CCL-185) were maintained at 37 °C with 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 mg/mL streptomycin sulphate. Human respiratory syncytial virus Long strain (ATCC VR-26) was propagated and titrated in HEP-2 cells (ATCC CCL-23).

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

 Antibodies and western blot - Total proteins were extracted by scraping and syringing cells in 1xNuPAGE LDS buffer (Invitrogen). Fifteen to thirty micrograms of total protein were then separated on 10% SDS-PAGE gels. The following antibodies were used: mouse monoclonal anti-Mdm2 (SMP14, sc-965, Santa Cruz Biotechnology), anti-p53 (DO-1, Santa-Cruz Biotechnology) and anti-Akt (#9272, Santa Cruz Biotechnology), rabbit polyclonal anti-Phospho Akt (cell473) (#4058, Santa Cruz Biotechnology) and anti-β-actin (#4274, Santa Cruz Biotechnology). In addition, an anti-Ku80 polyclonal antibody was used as a loading control (#2755, Cell Signaling).
Plasmid and siRNA transfection - Plasmid transfections were performed using TransIT-LT1 reagent (Mirus), according to the manufacturer’s instructions. NS1-EGFP and NS2-EGFP expression plasmids [46] were graciously obtained from Ralph Tripp (Department of Infectious Diseases, University of Georgia). p5V-p53 expression plasmid was a kind gift from Dr. Jean-Christophe Boudou (Division of Cancer Research, University of Dundee, UK). Silencing of p53 was performed in A549 cells transfected with a siRNA specifically targeting p53 (si-p53)[39] and a non-specific siRNA (si-Ctrl, OR-0030-nes05, Eurogentec), as a control, by using Oligofectamine (Invitrogen).

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

NF-κB and Cytokines/chemokines immunostaining:
Cell culture supernatants were analyzed for the presence of 27 human cytokines and chemokines, as previously described [47], using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Bio-Rad) on a FLEXMAP 3D® analyzer (Lumines, Austin, Texas, USA). The same technology was used for the monitoring of p65 phosphorylation, using the Bio-Plex Pro Phospho-NF-κB p65 kit (mp516) (BioRad).

Reagents. Small molecule Mdm2 antagonist Nutlin-3a (Calbiochem) was dissolved in DMSO, aliquotaged, and stored at −20 °C. A549 cells were infected by RSV at 1 MOI of 1, in presence of DMSO (Ctrl) or a small-molecule Mdm2 antagonist (Nutlin-3, 10 μM). Protein synthesis inhibitor cycloheximide (50 μg/mL) and proteasome inhibitor MG132 (20 μM) were purchased from Sigma-Aldrich (solf C7698 and M8699, respectively).
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Author contributions:
MD, PA, HI, TA, EH, LB, RCM, FBG and TO carried out the experiments and analysis of the results. RCM, FBG and TO participated in the conception and coordination of the study. MD, PA, RCM, FBG and TO designed the study and wrote the manuscript.

Conflict of interest:
The authors declare no conflict of interest.
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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 analyzed 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 form 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 μM cycloheximide (CHX), p53 protein stability was assessed during a 30-min period, by monitoring p53 relative protein levels (RPL). Mean values +/- 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 μM), as indicated.

Figure 3. RSV NS1 and NS2 both contribute to inhibit p53 transcriptional activity. A. A549 cells were transfected with 1 μg 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 μM. B. A549 cells were transfected with either an empty plasmid or two different amounts of plasmid expressing RSV NS1-eGFP (0.2 μg and 2 μg). 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 plasmids 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-eGFP or RSV NS2-eGFP (2 μg). At 48 h post-transfection, p53 protein stability was assessed by monitoring relative protein levels (RPL) of p53 during a 30-min period, after treatment with 50 μM cycloheximide (CHX). Mean values +/- 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 siRNA transfection using a control siRNA (si-Ctrl) or a specific siRNA (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 titers in supernatants (log10 TCID50/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. A549 cells were infected by RSV at a MOI of 1, in the presence of a small-molecule Mdm2 antagonist (Nutlin-3, 10 μM) or DMSO (Ctrl). The level of viral production was measured at 48 hpi using the two complementary methods described in A.

Figure S5. RSV infection modulates the p33/NF-kB balance via a 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-kB 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 μM). "", "**, 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 analyzed at 48 hpi. Cell lysates were analyzed by western blot for the expression of both total Akt and phosphorylated Akt. Ea50 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 analyzed by western blot for the expression of both total Akt and phosphorylated Akt. Ea50 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 a-ctrl or a-p53, were mock-infected or infected with RSV (Long strain) at a MOI of 1 and analyzed 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 (a-ctrl/a-p53). F. Model proposed for the role of RSV infection on the p33/NF-kB balance. RSV infection modulates the p53/NF-kB balance via the activation of the PI3K/Akt – Mdm2 pathway, mainly through the NS1/NS2 proteins. This alteration of NF-kB 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

A

HSV NS1

RSV NS2

E

C

D

Role of p35/NF-κB balance in RSV infection
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 remain 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

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

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

Commonly, an animal model of human respiratory infections includes small mammals like rodents because of their low cost, size, availability, the diversity of exploratory techniques and availability of reagents [10, 11]. To better understand the dynamics of host immune responses and pathogens the development of animal models with tissue interactions and immune system response homologous to those in humans is required. Insights of the mechanism of immunity and interactions with RSV or S. pneumoniae have been described in cotton rats [12-16] and mice balb/c [17-20]. Ferrets are among one of the best models to study influenza viruses, but RSV is able to replicate only in the upper respiratory system, or in the lungs of immunodepressed ferrets [30]. However, RSV is not a true infectious pathogen of small animals (mice and ferrets) being semi- to not permissive to the infection and limiting virus replication in lung tissue. Additionally, immune response differences between these animals and humans are relevant.

Mixed infections were showed to correlates to the severity of pneumonia and in vivo study described that viral respiratory infections can enhance pneumococcal severity [31]. Also, S. pneumoniae has been shown to enhance RSV infection in differentiated human airway epithelial cells, in vitro, and in cotton rats [32]. Thus, correlations between RSV and S. pneumoniae
infection were already described in the literature but more information concerning disease
development and severity is needed.

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

In other nonhuman primates like bonnet monkeys [45-47], owl monkeys [40, 48-50], Cebus
monkeys [31] and Infant baboons [52] some groups have described RSV infection, but always
requiring high doses of virus and few or no clinical signs of the disease [53]. The feasibility to
mimic pneumococcal pneumonia in macaques has already described elsewhere [54],
showing that intranasal injection in infant rhesus macaques resulted in symptomatic
pneumococcal pneumonia after 48 hours.

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

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

In order to develop the model of severe pneumonia in NHP, each macaque was infected as
described in table 1 and monitored during 23 days to evaluate the symptomatic infection of SP
and RSV. To characterized pneumonia and disease severity, bronchoalveolar lavage (BAL) fluids, upper respiratory swabs, and blood samples were collected throughout the 23 days of infection and analyzed for bacterial culture and quantification.

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

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

Blood was also analyzed in complete blood count to identify inflammation markers. During a bacterial single infection, we were able to detect monocytosis (Fig. 1A) and lymphopenia was found in RSV single and mixed infected. No differences in the white blood cell count were identified by Kraft et al. even in high doses of bacteria group like in our results. However, they described leukopenia after 48 hours which we don’t detect but differences in quantities of
bacteria inoculated can explain these differences [54]. Another study was already showed that the proportion of neutrophils in BAL of monkeys infected with RSV increased only to 9%, [45], whereas neutrophils are the predominant cell in RSV infected children [57].

Weight, temperature, and behavior were also analyzed and there was no difference between the control and the infected ones. Clinical signs of disease are uncommon following RSV infection in NHP model, and they develop mild histopathological changes in the lung. Another important contribution to detect the development of pulmonary lesions and track the course of illness can be the use of thoracic imaging [38].

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

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

Mixed infection in our model did not progress to severe pneumonia. We believe that the health status of the animals protected them from more severe disease, similar to healthy humans infected with RSV and carriage of Sp. Despite this, mild pneumonia was found in our model. The severity of pneumonia is dependent upon the viral and bacterial strain used, pathogen dose, and route of infection and maybe high-dose of each pathogen is needed to induce more than mild illness in
primates. Another strategy for severe pneumonia model could be to develop immunodeficient NHP. To expand the relevance of our findings and establish the utility of this model for the prevention and treatment of severe pneumonia, additional experiments are needed. In perspective, NHP model will help to describe several mechanisms involved in the pathogenesis of co-infection and identify prognostic biomarkers of severe pneumonia. Also, this model is important to validate findings in small animal models and may more closely predict vaccine and therapeutic efficacy for severe disease in humans.
Figure legends

Table 1. Pathogens infection distribution by animal. Cynomolgus macaques (*Macaca fascicularis*) were acclimatized to laboratory conditions for one month prior to first treatment. Then, RSV and/or Sp infected (n=4) and pathogen-free macaques (n=2) was inoculated with $1.10^7$ TCID$_{50}$/mL of RSV-A and $1.10^6$ CFU/mL of *S. pneumoniae* serotype 14 intranasal and intratracheal to maximize lower respiratory tract disease.

Figure 1. RSV and/or Sp infections: study design and results. A) Timeline showing infections time points, days of samples collection and lung histopathology. Colored bars resume analyzed results of all methodologies utilized. Blue bars represented lymphopenia, brown bars represented monocytosis and red bars low hemoglobin, erythrocytes and red blood cells. Samples positive for *S. pneumoniae* by PCR are shown in respective days of detection by purple bars. B) Histopathology of the lung of all infected animals showing *S. pneumoniae* infected an animal with slight signals of inflammation, RSV-infected characterized by lymphoid clusters and two co-infected animals showing areas of sub-pleural reinfece (01) and congestive vessels, inflammatory cells, and hemorrhage (02).
<table>
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<th>Experimental group</th>
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<td>RSV (TCID50/mL)</td>
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<td>Animal 2 – Non-infected</td>
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<td>Animal 6 – Mixed-infected</td>
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DISCUSSION AND PERSPECTIVES
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-kB). 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-kB 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 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 para-influenza 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.
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 Δ133p53α and p53β in the context of an IAV infection [396, 402]. In the literature, it was reported that p53β can promote p53 transcriptional activity while Δ133p53α 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 Δ133p53α and p53β isoforms were described in the literature, showing a modulation of the expressions of Δ133p53α and p53β 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 Δ133p53α and p53β expression.

Early stages of infections

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
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 dérègule 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 pathogeneses, 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.
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 p33 pathway has been reported in several studies, highlighting the antiviral contribution of p33. Here, we investigated the impact of IAV on the E3-ubiquitin ligase Mdm2, a major regulator of p33, and observed that IAV targets Mdm2, notably via its non-structural protein (NS1), therefore altering Mdm2 stability. p33/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 p33-independent functions. Alas, our work contributed to better understand the mechanisms underlying the complex interactions between IAV and the p33 pathway, for which both NS1 and Mdm2 arise as key players.

Keywords

Mdm2; E3-ubiquitin ligase; p33; apoptosis; influenza virus; virus-host interactions
Introduction

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

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

Mdm2, considered as the main negative regulator of p53, is an RING-finger E3 ubiquitin ligase that binds to p53 to promote its ubiquitination and degradation. Mdm2 and p53 participate in a negative feedback loop wherein p53 activates the transcription of Mdm2, which in turn inactivates p53 by directly associating with it and promoting its ubiquitination and proteasomal degradation. Since p53 is largely regulated at the post-translational level, the possible alteration of the Mdm2–p53 interaction, decrease of Mdm2 protein levels and/or subcellular delocalization of Mdm2 have been suggested as the primary mechanisms for stabilization of p53. Nevertheless, only a limited number of studies have investigated the role of Mdm2 in the context of infection by non-oncogenic viruses, such as
hantviruses for example. Although two different genome-wide RNAi studies, both performed in
human lung epithelial cells, have highlighted Mdm2 as a crucial factor for IAV infection, the role of
Mdm2 in IAV infection remains unclear. In that regard, Wang and collaborators have recently shown
that the stabilization of p53 in IAV infected cells was associated with a compromised Mdm2-mediated
ubiquitination of p53.

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

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

Since subcellular localization is known to play a major role in Mdm2 activity towards p53, and to further investigate the impact of IAV infection on Mdm2, we performed immuno-fluorescence confocal microscopy in A549 cells mock-infected or infected by influenza A (H3N2) virus at 8 or 24 hpi (Fig. 2). In addition to specific Mdm2 immunostaining (red), we also used IAV non-structural protein NS1 (green) as an indicator of viral cycle advancement. In mock-infected cells, Mdm2 is mainly localized in nuclei (Fig. 2, panels a and a). In IAV-infected cells, Mdm2 immunostaining signal was slightly increased at 8 hpi, compared to mock-infected cells, with a visible nuclear accumulation (Fig. 2, panels i and j), and a co-localization with NS1 in several cells (Fig. 2, panels 1 and p). At late stages of infection (24 hpi), we observed a stronger increase of global Mdm2 staining, yet with partial localization in the cytoplasm (Fig. 2, panels q and q) as well as a relative exclusion between Mdm2 and NS1 respective stainings (Fig. 2, panels t and t). These observations confirmed that IAV strongly impacts endogenous Mdm2 protein levels, but also alters its nucleo-cytoplasmic localization.

IAV infection modulates Mdm2 stability.

We then investigated the impact of IAV infection on Mdm2 stability. To that end, we mock-infected or infected A549 cells in specific conditions, reproducing a scenario of marked decrease (MOI 4, 4 hpi) or increase (MOI 0.1, 24 hpi) of Mdm2 RPL and analyzed Mdm2 stability by monitoring protein levels over a 1 h period after treatment with 50 µM of cycloheximide (CHX) (Fig. 3A and Fig. 3B). At early stages of infection (4hpi), we observed a faster decrease of Mdm2 RPL on infected versus mock conditions, with an estimated half-life of 12 and 30 min, respectively (Fig. 3A). Conversely, Mdm2 estimated half-life was almost 3 times higher in IAV-infected cells compared to mock (60 versus 20 minutes, Fig. 3B) at late stages of infection (24hpi). We then further investigated the decrease of Mdm2 at early stage of infection (4hpi). We observed less pronounced IAV-induced destabilization of Mdm2 at 4 hpi in the presence of proteasome inhibitor MG132 (Fig. 3C). Moreover, ubiquitination assay in IAV-infected cells revealed an increase on Mdm2-ubiquitin conjugates between 0 and 6 h post-
infection (Fig. 3D). Altogether, these results indicate that IAV infection modulates Mdm2 stability, with a role of ubiquitin-dependent proteasomal degradation at early stages of infection.

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

We previously demonstrated that IAV non-structural protein NS1 contributes to p53 stabilization. 13 Although the underlying mechanism was not fully understood, Wang and collaborators proposed that IAV-induced p53 stabilization was associated with a compromised Mdm2-mediated ubiquitination of p53 19. In an attempt to shed light on the potential role of NS1, we transfected A549 cells with either an empty plasmid or a plasmid expressing the influenza A (H1N2) NS1 protein and assessed Mdm2 stability as described above but over a 4 h period (Fig. 4A). Compared to mock-transfected cells, we observed a significant decrease of Mdm2 RPL in NS1-expressing cells at 40 and 60 min time-points (p< 0.001, Fig. 4A). Indeed, the estimated Mdm2 half-life in the context of transient expression of NS1 was almost the half of that observed in mock conditions (35 versus 75 min, Fig. 4A). In parallel, we also observed that transient expression of NS1 (data not shown).

We then hypothesized that NS1 could constitute a major determinant involved in virus-induced destabilization of Mdm2. To further explore the potential contribution of NS1, we used reverse genetics to produce 4 recombinant influenza viruses sharing the same A/Peru1951/8/34 (H1N1) genomic background, but harbouring the NS1-coding segment (NS) issued from different IAV strains and subtypes: NS from A/Peru1951/8/34 (H1N1, PR8), swine-origin A/Nebraska/09/09 (H1N1, SO), A/Moscow/10/99 (H3N2, MO), A/Frankfurt/England/20151/94 (H5N2, EN). A549 cells were mock-infected or infected with the different recombinant IAV at a MOI of 4 and cell lysates were harvested at 8 hpi for western blot analysis (Fig. 4E). The four recombinant IAV induced significant yet differential decrease on Mdm2 RPL (p<0.0001) at a similar stage of infection, as confirmed by NS1 protein levels. Indeed, NS1 issued from H3N2 and H5N2 strains appeared to induce Mdm2 destabilization more efficiently than their H1N1 counterparts (PR8, SO), suggesting a possible strain/subtype specificity in this process (Fig. 4B). In addition, we also evaluated the impact of NS1
expression on the interaction between p53 and Mdm2 by transfecting A549 cells with either an empty
plasmid, or two different amounts of plasmids (1 and 4 µg) expressing NS1 cloned from the H3N2
strain (Fig. 4C). After 48 h post-transfection, cell lysates were analyzed by co-immunoprecipitation
assay using an anti-p53 polyclonal antibody. As expected, in the absence of NS1, Mdm2 was co-
immunoprecipitated with the anti-p53 antibody. In contrast, the transient expression of NS1 sharply
decreased Mdm2 co-immunoprecipitation, correlated with decreased Mdm2 protein levels in the input
(Fig. 4C). In conclusion, our results underline NS1 as a key determinant in the IAV-induced
destabilization of endogenous Mdm2, at an early stage of infection.

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

To further investigate the role of Mdm2 in IAV infection, we first evaluated the impact of Mdm2
knockdown on IAV production. A549 cells were transfected with either a non-specific siRNA (si-Ctrl)
or a pool of siRNAs targeting Mdm2 and/or p53. Forty-eight hours post-transfection, cells were
infected with influenza A (H1N2) at a MOI of 0.01 and viral supernatants and cell extracts were
harvested at 24 hpi. As expected, western blots confirmed that p53 and/or Mdm2 expression levels
were reduced in cells transfected by specific siRNAs targeting p53 and/or Mdm2, respectively,
compared to si-Ctrl treated cells (Fig. 5A). Moreover, viral production at 24 hpi was assessed by three
different approaches: (i) quantification of influenza M genome copies by RT-qPCR, (ii) measure of
infectious viral titres by endpoint titration in supernatants, and (iii) evaluation of NS1 protein levels by
western blot. In agreement with previously published work validating the antiviral role of p53, we
observed that viral genome copy number was significantly increased (approximately 10-fold) in
supernatants of si-p53 transfected cells, compared to that of si-Ctrl transfected cells (Fig. 5A). This
result was confirmed by both infectious titres, with more than 10-fold increase on TCID50/mL under
si-p53 treatment compared to si-Ctrl, and increased NS1 protein levels in western blot (Fig. 5A).
Based on these initial observations, and considering Mdm2 is mainly recognized as a negative
regulator of p53, we anticipated to observe the opposite effect with si-Mdm2. Surprisingly, we
obtained very similar results between si-p53 and si-Mdm2 experimental conditions, independently of
the readout used (Fig. 5A). For example, we observed that viral genome copy number was
significantly increased (more than 10-fold) in supernatants of si-Mdm2 transfected cells, compared to
that of si-Cul transfected cells (Fig. 5A). Interestingly, in the case of a combination of si-p53 and si-
Mdm2 treatment, we observed a strong decrease of viral production compared to si-Cul, with more
than 100-fold decrease in viral genome copies/mL and TCID50/mL, but without a marked difference
of NS1 protein level in western blot (Fig. 5A). To complete these results, we performed a minor
experiment, using a transient expression approach in H1299 cells (Fig. 5B) that not only lack the
expression of full-length p53 but also express Mdm2 at low levels. Cells were transfected with either
an empty plasmid or a plasmid expressing p53 or Mdm2, or alternatively co-transfected with plasmids
expressing p53 and Mdm2. Forty-eight hours post transfection, cells were further infected with
A/Moscow/10/99 (H3N2) virus, and the impact of transient expression on viral production was
assessed by measure of infectious titres, viral genome copy numbers and viral protein level (Fig. 5B).
The transient expression of p53 and/or Mdm2 was confirmed by western blot. As expected, viral
production was significantly lower in cells transfected with p53 (p<0.01) compared to mock-
transfected cells, in line with our previous observations 10. Interestingly, a similar impact of Mdm2
transient expression was observed, with almost 100-fold decrease on both genome copies/mL and
tCID50/mL titres compared to mock-transfected cells, consistent with a marked decrease of NS1
protein levels in western blot (Fig. 5B). The co-transfection of p53/Mdm2 was also associated with a
decrease of viral production.

To further investigate the role of Mdm2 in IAV infection we used small Mdm2 antagonist molecules,
such as Nutlin-3 21 or NSC6681 22, which are known to bind Mdm2 in its p53-binding region,
blocking Mdm2 regulatory activities towards p53, and hence inducing the stabilization and activation
of p53 23. We therefore evaluated the impact of these two Mdm2 antagonists on IAV production, by
infecting A549 cells with influenza A/Moscow/10/99 (H3N2) virus at a MOI of 0.001, in the presence
of either DMSO (control) or different concentrations of Mdm2 antagonists (Fig. 5C). Cells were pre-
treated 14 h before infection to ensure good efficacy of Mdm2-antagonist treatment, and harvested at
48 hpi. Treatment with Nutlin-3 significantly increased viral production compared to control, with up
to 40-fold increase of log10 RNA copies/mL for 10 μM (p<0.05) (Fig. 5C). A similar yet milder effect
was observed for the NSC66811 molecule, with 5-fold maximum increase on viral production for 2 μM (p<0.001). Of note, the same impact of small molecule antagonists on IAV production was observed in absence of p53, in H1299 cells, using lower doses of antagonists or shorter viral kinetics (data not shown). These results are consistent with our previous observations using si-RNA and transient expression approaches, suggesting a mostly p53-independent antiviral role of Mdm2.

We then explored cellular processes regulated by p53/Mdm2 and therefore potentially impacted by Mdm2 antagonists. One striking observation is the impact of Nufin-3 and NSC66811 on the early steps of IAV-induced apoptosis (Fig. 5C). We infected A549 cells by influenza A (H3N2) at different MOIs for 48h, in presence of control DMSO or Mdm2 antagonists at different concentrations, and using a specific luciferase assay to measure caspase 3/7 activity at 48 hpi. As expected, whereas higher MOIs were correlated with an increase of caspase 3/7 activity in DMSO-treated cells (control), the impact of IAV infection on apoptosis was strongly reduced, or almost completely attenuated in the context of Nufin-3 or NSC66811 treatment (Fig. 5C). These results suggest a possible reduction of IAV-induced apoptosis by Mdm2 antagonists, creating a favourable context for IAV replication.
Discussion

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

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

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

Different experimental approaches in our study suggest that the p53/Mdm2 autoregulatory negative feedback loop is altered during the time course of IAV infection, notably at late stages (Fig. 1 and Fig. 3). This kind of modification has been already described in literature in different contexts of cellular
stressed stimuli, such as DNA damage, oncogenic or nuclear/ribosomal stress\(^{26,17,17}\). Since we previously demonstrated that IAV infection induces a strong remodelling of the host nucleolus\(^{38,39}\), we cannot exclude a role of nuclear/ribosomal stress in IAV-induced alteration of the Mdm2/p53 loop.

Another non-exclusive hypothesis would be the involvement of viral proteins such as NF and NS1 in this mechanism, as we have recently demonstrated their functional interaction with host nucleolus and ribosome machinery at different levels\(^{28-30}\).

Our results show that NS1 contributes to Mdm2 degradation either directly or indirectly (Fig. 4), which is in line with our previous work on the induction of p53 stabilization by transient expression of NS1\(^{15}\). We then can hypothesize that NS1 might contribute to p53 stabilization at different levels, via its interaction with p53 and/or its contribution to Mdm2 degradation. Although the underlying mechanisms remain to be investigated, several observations might be worth exploring. For example, our immuno-fluorescence confocal microscopy results (Fig. 2) indicate a progressive decrease of co-localization of Mdm2 and NS1 during the time course of infection, in correlation with the stabilization of Mdm2 observed at late stages of infection (Fig. 1 and Fig. 3), which suggests that NS1 contribution to Mdm2 degradation could occur in a nuclear compartment. Interestingly, NS1 has been shown to target other E3-ubiquitin ligases such as RING-domain containing TRM proteins involved in innate immunity\(^{45}\), for which we cannot exclude a similar mechanism in the case of Mdm2. In addition, the differential effect of NS1 on endogenous Mdm2 in the context of infection by different recombinant IAV (Fig. 4B) suggests a virus strain/subtype specificity, which could be linked to specific NS1 amino-acid differences that may impact the interaction, subcellular localization and/or functionalities of NS1. In addition, NS1 from different IAV strains/subtypes were shown to differentially activate the PI3K/Akt signalling pathway\(^{38,10}\), which is part of the upstream regulatory pathway of Mdm2\(^{17}\). The possible role of NS1 functional interactions with upstream signalling pathway (MAPK, ERK, PI3K/Akt) and their consequence of Mdm2 underscores further validation.

The most striking result of our study is the unexpected inhibitory role apparently played by Mdm2, as suggested by silencing/transient expression experiments as well as small molecule Mdm2-antagonists (Fig. 5). This potential antiviral facet of Mdm2 is quite interesting, as Mdm2 is the main negative regulator of p53\(^{17}\) and p53 is known to contribute to the antiviral response\(^{33,14}\). However, several
results indicate that Mdm2 limits IAV production in a p53-independent manner (Fig. 5A). Indeed, Mdm2 has been reported to have p53-independent functions in a large panel of cellular processes such as cell cycle control, cell fate determination, or signalling pathways (e.g. NFκB) \(^{5,36}\), all of which could be involved in the property of Mdm2 to inhibit IAV infection.

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

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

Viruses and cells – IAV strain A/Moscow/10/99 (H3N2) and recombinant viruses used in this study were cultivated and titrated in MDCK cells and stored at –80 °C, as previously described 16,18. MDCK cells were purchased from Lonza (ATCC, CCL34) and were passaged twice weekly in serum-free Ultra-MDCK medium (Lonza) supplemented with 2 mM L-glutamine (Sigma Aldrich), penicillin (225 units/ml) and streptomycin (225 µg/ml) (Lonza). Human lung epithelial A549 cells (ATCC CCL-185, wild type p53) and H1299 cells (ATCC CRL-5803, Homozygous partial deletion of TP53 gene) were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM, Lonza, Biehwittaker) supplemented with 100 units/ml penicillin, 200 µg/ml streptomycin, 2 mM L-glutamine and 10% fetal calf serum (Dutcher). All cells were maintained at 37 °C with 5% CO2. Sub-confluent A549 or H1299 cells were infected with influenza viruses at different multiplicity of infection (MOI, indicated in text and figure legend). After a 1h adsorption period in a minimal volume, DMEM supplemented with 10% heat-inactivated fetal bovine serum (Lonza), L-glutamine (3mM), penicillin (100U/ml), streptomycin (200 µg/ml) and 0.5 µg/ml trypsin was added, and cells were incubated at 37°C for different lengths of time. Mock-infected controls were realized with the same protocol, using DMEM instead of viral inoculum.

Reverse genetics – Reverse genetic system A/PR/8/34 (H1N1), and all NS reassortant viruses were generated by reverse genetic as previously described 18,19. 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/Indiana/096/09 (H1N1, SO), A/Moscow10/99 (H5N2, MO), A/Finch/England/2011/94 (H5N2, EN). Recombinant viruses were generated by DNA transfection. Plasmids were mixed with Superfect reagent (Qiagen) in OptiMEM (GIBCO), according to the manufacturer’s instructions, and added to 293 T cells in six-well tissue culture plates. At 48 h post-transfection, viruses in the culture supernatant were harvested and used to infect MDCK cells to be amplified. After two passages, viral titers were measured using standard methods.
**Immunofluorescence confocal microscopy.** A549 cells grown, and Mock or IAV-infected on Lab-Tek II chamber slides (ThermoScientific) were fixed with 4% paraformaldehyde in PBS for 30 min. After washing in PBS, cells were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 15 min. Mouse monoclonal anti-Mdm2 antibody (SMP14, sc-965, Santa Cruz Biotechnology), and a rabbit polyclonal anti-NS1 (Kind gift of Dr. Juan Ortin, CSIC, Spain) were used as primary antibodies in PBS-T. After incubation for 1 h, the cells were washed in PBS-T and then incubated with goat anti-mouse coupled to AlexaFluor 633 and/or goat anti-rabbit coupled to AlexaFluor 488 (Molecular Probes, Invitrogen) for 30 min, at concentrations recommended by the suppliers. Nuclei were counterstained with DNA-binding fluorochrome 4,6-diamidino-2-phenylindole (DAPI, Invitrogen). After staining, coverslips were mounted with Fluoromount G (Cliniscience) and analyzed using a confocal laser scanning microscope (SP5 Leica).

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

**Stability, transactivation and RT-qPCR assays.** For the determination of Mdm2 half-life, cells were treated with cycloheximide (50 μg/mL), and total protein lysates were harvested at different time points and analyzed by western blot. Mdm2 relative protein levels (RPL) were determined by densitometry analysis using the ImageJ software (http://rsbweb.nih.gov/ij/). For transactivation assays, cells were transfected with 1μg of Mdm2-luc vector, possessing the firefly luciferase gene under the control of the partial promoter sequence of Mdm2 (Mdm2-luc) 39. Transfection efficiency was normalized using a Renilla Luciferase plasmid. Luciferase activity was measured in whole cell
extracts using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer’s instructions, and was expressed as Relative Luciferase Units (RLU), compared to the control. For real-time quantitative PCR, total RNAs were extracted using the RNAeasy Mini Kit (Qiagen). Reverse-transcription was performed on 1 μg of total RNAs using the Superscript II enzyme (Invitrogen) at 42°C. Quantification of the levels of the different mRNAs of interest was performed by real-time quantitative PCR, as previously described (Terrier et al., 2011).

Immunoprecipitation & Purification of His-tagged ubiquitin conjugates – For immunoprecipitation, AS49 cells were mock-infected or infected at different MOI. Cells were harvested 8 or 24 h post infection in a NP40 lysis buffer (NP40 (1%), NaCl (150 mM), Tris-HCl (20 mM)). Lysates were incubated with an anti-p33 polyclonal rabbit antibody (CMI, Novocastra Laboratories) on a rotating wheel overnight at 4°C. Protein G agarose beads (Life technologies) were then added to the lysate and incubated 1 h at room temperature. The beads were pelleted and washed in NP40 lysis buffer.

Antibody-antigen complexes bound to the beads were eluted in 1X NuPAGE LDS buffer (Invitrogen) and analyzed by western blot with an anti-Mdm2 antibody (SMP14, Santa Cruz biotechnology). For purification of His-tagged ubiquitin conjugates, cells grown in 150 mm dishes were transfected with a plasmid expressing his-tagged ubiquitin (pUb(His)6). The purification of His-tagged ubiquitin conjugates was performed as previously described 44. Briefly, cells were harvested in a lysis buffer (Guanidinium-HCl (3 M), NaH2PO4 (0.1 M), Tris-HCl pH 8 (0.01 M), Tween 20 (0.05 %)). Lysates were mixed with Ni-NTA agarose beads (QIAGEN) and incubated on a rotating wheel 2 h at room temperature. Beads were then washed (Urea 8 M, NaH2PO4 (0.1 M), Tris-HCl pH 6.3 (0.01 M), Tween 20 (0.05%)) and His-tagged ubiquitin conjugates were eluted (Imidazole (200 mM), Tris-HCl pH 6.7 (0.1 M), glycerol (30%), β-mercaptoethanol (0.72 M), SDS (5%)). The eluates were analyzed by western-blot with the appropriate antibodies.

Plasmid and siRNA transfection - Plasmid transfections were performed using TransIT-LT1 reagent (Mirus), according to the manufacturer’s instructions. The Ha6-tagged ubiquitin expressing construct was a kind gift from Dr Mark Saville (Division of Cancer Research, University of Dundee, UK). The
NS1 (H3N2) plasmid (pCI-NS1 H3N2) was a kind gift from Dr Nadia Naifakh (Pasteur Institute, France). Silencing of Mdm2 was performed in A549 cells transfected with the Smart Pool ON-TARGET plus Mdm2 (si-Mdm2) (L-003279-00-0005, Thermo Scientific), a siRNA specifically targeting p53 (si-p53) and a non-specific siRNA (si-Ctrl, CR-0030-NEG05, Eurogentec), using Oligofectamine (Invitrogen).

Small molecule Mdm2 antagonists - Two different small molecule Mdm2 antagonists were used in this study, Nutlin-3a (Calbiochem) or NSC66811 (Calbiochem). A549 cells were pre-treated for 14h, and then 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.
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Conflict of interest

OT, JCB and MRC are co-inventors of a patent application deposited by University of Dundee, Centre National de la Recherche Scientifique, Université Claude Bernard Lyon 1 and Hospices Civils de Lyon (FR20100058132 20101105; WO2011FR52575 20111104). The other authors declare that they have no competing interests.
References:


Figure legend:

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 24 hpi, 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 b) 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, r, w). Merged fluorescent signals are presented in panels d, h, i, p, t and x. Cell details are enlarged (inset). White scale bar = 10 μm.

Figure 3. IAV infection modulates Mdm2 stability. Stability assay in IAV-infected cells at 4 hpi (A) and 24 hpi (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 24 hpi, respectively. Stability was assessed by monitoring relative protein levels (RPL) of Mdm2 during a 1 h period, after treatment.
with 50 μM cycloheximide (CHX). Mean values +/- 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 μM), 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 μM 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 1 h period, after treatment with 50 μM cycloheximide (CHX). Mean values +/- 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/069/09 (H1N1, SO), A/Moscow/10/99 (H3N2, MO), A/Finch/England/20151/04 (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 μg 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-RNA (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 titer of supernatants (log_{10} TCID50/mL) by endpoint titration in MDCK cells (measured in triplicate in 2 independent experiments; ** for p-value < 0.001), (ii) RT-qPCR (log_{10} RNA copies/mL, measured in 3 independent experiments), and (iii) western blot. Knu80 was used as a loading control. B. Transient expression/co-expression of p53 and/or Mdm2 in H1N19 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 6831). 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 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)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 2,204 (H1N1)pdm09 hemagglutinin (HA) gene sequences collected during and after the pandemic period (2009-2014). In addition, we have looked for evidence of viral polymorphisms associated with antigenic drift and compared the range of viral variants with the vaccine strain (A/California/07/2009) used throughout this period. The phylogenetic analyses in this study revealed the circulation of at least eight genetic groups in Brazil. Two (GI-5 and C7-6) co-circulated during the pandemic period, showing an early pattern of viral diversification with low genetic distance from vaccine strain. Other phylogenetic groups, C3, C6 (including GI-6, C4 and 60 subgroups), and C7 were found in the subsequent epidemic season 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 vaccine efficacy. Furthermore, we observed that the presence of any polymorphism at residue 222 of the HA gene was significantly associated with several-fold increase, reinforcing previous reports that described the residue as a potential virulence marker. This study provides new information about the circulation of some viral variants in Brazil follows up potential genetic changes associated with virulence and allows infer if the efficacy of the current vaccine against more recent (H1N1)pdm09 strains may be reduced.

1. Introduction

Influenza viruses are important human pathogens, causing seasonal epidemics and occasional pandemic 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 (Diamond et al., 2009; Smith et al., 2009). This virus was the product of multiple re-occurrence events involving viruses from swine, birds and humans and was responsible for more than 15,000 deaths worldwide (Smith et al., 2009). 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 (Diamond et al., 2009; Smith et al., 2009). 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 4.8% among severe acute respiratory infections (SARI) cases were reported (Secretary of Health Surveillance – Brazil, 2010). After the pandemic period, H1N1pdm09 began to circulate as a seasonal virus together with the seasonal influenza A (H3N2) and influenza B virus lineages, causing annual epidemiologic seasons during winter and considerable morbidity and mortality (Secretary of Health Surveillance – Brazil, 2012; Secretary of Health Surveillance – Brazil, 2013; Secretary of Health Surveillance – Brazil, 2014).

Influenza viruses have three 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 (Carst and Flahault, 2007; Medina and Garcia-Saute, 2011). It contributes to the ability of the virus to evade recognition by the immune system and vaccine consistent circulation within human populations (Carst and Flahault, 2007; Medina and Garcia-Saute, 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
A, H1N1 and H3N2 and one influenza B lineage. Since the emergence of A/H3N2 pdm09, the H3N2 vaccine component has retained the same in the Northern and Southern Hemispheres (World Health Organization, 2014) 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 the recipient or with the virus strain chosen to compose the anti-influenza vaccine (Ko et al., 2015). The intrinsic capacity of the recipient to respond to the vaccine strain, for example, may be modulated by many characteristics such as age, sex, nutritional and immunological conditions, genetic factors, and presence of chronic disease (Lavazere-M坚持, 2001). Influenza vaccine effectiveness is mostly affected by the high mutation rate of influenza viruses. Effectiveness figures of 70–80% against influenza have been reported, depending on the antigenic match between vaccine and circulation strain (Noyel et al., 2006; Ho and Swayne, 2015). The rate of amino acid change of influenza is 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 vaccinated. Studies have attempted to evaluate the use of sequence-based amino acid changes to predict these antigenic changes (Gleis et al., 2010; Lee et al., 2010; Mesquita et al., 2014; Resende et al., 2014; Souto 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 (Gleis et al., 2010; Lee et al., 2010; Mesquita et al., 2014; Resende et al., 2014; Souto et al., 2013). The HA gene is the major viral antigens 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 viruses have been suggested as potential virulence markers, as K22E and Q59I (Gleis et al., 2010; Poled et al., 2010). D226G (World Health Organization, 2013). However, the substitution of aspartic acid (D) to glycine (G) or mixed viral populations at residue 22 of the HA gene presented a strong association with severe and fatal cases (Chen et al., 2010; Erande et al., 2010; World Health Organization, 2010b; Resende et al., 2014). Regarding the phylogenetic dynamic of the A/H1N1 pdm09 virus, previous studies showed an 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; Poled 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/H1N1 pdm09 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. Materials 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 Health Organization (WHO) and for the Ministry of Health (MoH), where this study was conducted. A total of 20 representative Brazilian A(H1N1)pdm09 strains were chosen for this study. These selected samples were representatives of the viruses circulating in the three distinct Brazilian geographic regions, namely, Northeastern (n = 31), Southeastern (n = 17) and Southern (n = 145) covering the epidemiological season (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 all individuals ranging from 15 to 39 years-old, and 54% were females. Among these patients, only 13% (62/473) provided detailed information about clinical outcomes, 44% cases were mild, 67 were severe and 37 were from fatal cases, and only 14 patients reported chronic disease. Samples were manipulated anonymously. Patient identifiers including personal information (names and addresses) and hospitalization number were removed to protect patient confidentiality. This study was conducted with 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 purpose of the study, a formal approval from the Institutional Committee was not required.

2.2. DNA extraction, amplification and sequencing of the HA gene

Viral RNA was extracted from NPAs using QIAamp viral RNA mini kit (Qiagen, Hilden, Germany) according to manufacturer protocol. cDNA was synthesized using Super Script II enzyme (Life technologies, Carlsbad, California, USA). Thereafter, whole HA sequence was obtained by amplification of full PCR products using tag Platinum DNA polymerase enzyme (Life technologies, Carlsbad, California, USA) and primer set 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 3130 Genetic Analyser (Applied Biosystems, California, USA). Sequences were assembled and the comparison with the reference sequence (A/California/7/2009) were performed using Sequence software 51 (Gene Codes, Ann Arbor, MI). Mixed nucleotide polymorphisms at residue 22 were found and the sequencer method was chosen using a custom pyrosequencing (PSQ) protocol described previously (Levine et al., 2011). Brazilian sequences were submitted to the Global Initiative on Sharing Influenza Data (GISAID) database and are available with the accession numbers: EF178265 to EF178485.

2.3. Data set construction, genetic and phylogenetic analyses

A data set with 286 HA sequences was constructed, including Brazilian sequences (n = 230) 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 (C1, C2-pdm to C7-pdm) and post-
pandemic periods (G2 to G7, including 6A to 6C subgroups) (European Centre for Disease Prevention and Control, 2011, 2011; Nelson et al., 2009; Pastur 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 Generius 7.0 software) and edited manually for optimization.

Maximum likelihood (ML) trees were inferred with the on-line PhyML platform (http://www.atgc-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 NodeTest 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 > 0.5 and the tree was rooted with the vaccine strain A/Cambria/7/2009.

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

Amino acid substitutions from Brazilian H1N1 sequences were compared with vaccine strain using Sequencer 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 $E = 0.47 - 2.47 \times p_{max}$ described by Demm and Jan (2005). $p_{max}$ 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, Sr, Ca, G2, and Cb.
of the HA1 globular domain, against which the majority of neutralizing antibodies are directed (Ganem et al., 2016).

2.5. Statistical analysis

The software BATTS (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 statistic 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 Metagenes software (Parker 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/coningrv1/). Statistical significance was considered only for p-values < 0.05.

3. Results

3.1. Phylogenetic analysis 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 morphological 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 period: G3-pdm, whose sequences clustered closely with A/New York/47/2009-like with K-13C and Q358H substitutions and G5-pdm, similar to A/HongKong/1/2009-like with S237T substitution. Four Brazilian strains (A/BH/29/ 6203/2009, A/BH/29/2009, A/BH/29/2005, A/BH/29/2005, A/BH/29/2005, A/CM/202/2000, A/M/202/2000, A/M/202/2000) were not clustered into G3-pdm and G5-pdm. Since this was the whole genome to classify the other early phylogenetic groups from pandemic period, the 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. In May 105 strains analyzed from the large majority (87.7%) were G3-pdm, followed by the G5-pdm (12.3%). The G7-pdm, was the origin of later phylogenetic groups (G2 to G7) identified in the post-pandemic period, they are slightly aligned at the ML tree (Fig. 1A, indicated by arrows). Post-epidemic 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 G5 and G7 in 2013 (Fig. 1B and C). Only the G6 subgroup 6B was detected in 2014 epidemic season (Fig. 1D). G6 and G7 variants were less common than G6. The G5 A/Estrela/1/2011-like sequences were characterized by D97N, E208R, E210D and Y246H substitutions. Twenty-three Brazilian strains clustered in this group, were collected from May 2011 to August 2012 (Fig. 1B). Seventeen C7 Brazilian sequences detected in 2012 and 2013 seasons were A/ St Petersburg/100/2011-like, being characterized by S134T, S197T and A197T signature substitution. In addition to these signatures, strains from 2013 also exhibited the L-M, S46K and K163S substitutions (Fig. 1C). Regarding G6, represented by strains A/Saint Petersburg/27/ 2011-like with the E99N and S180T substitutions, it has circulated widely in Brazil since 2011, represented by the strains A/Brazil/279/ 2011_May and A/Brazil/301/2011_Jun (Fig. 1D), and more than half of Brazilian isolates (n = 116) analyzed in this study belonged to it. This group also shows the wider diversity, being divided in four subgroups, 5A to 60. The subgroup 6B was the most populated enclaving 79 Brazilian sequences from 2013 to 2014 and the signature substitutions K163S and A256T. The G6 phylogenetic subgroup (n = 33), was defined by the N50T mutation. Only two samples were classified as subgroup 6C at this collection and not one sample was clustered as subgroup 6D.

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 BATTS 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. 1A and Fig. 1B). The substitutions of D to G and/or asparagine (N) at residues 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 outcomes reported. The polymorphism E222G 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 PCR protocol on residue 222 of the HA gene. This protocol allowed us to detect in RRT codons, one strain with D222G/C mutations and five with D222N/ C mutations, and in GRT codons, one strain with D222G/C among the deceased patients, four were pregnant women and all presented mixed viral population.D222G (n = 1) and D222N (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 site). They were distributed in all phylogenetic groups, except in ungrouped-pdm. Groups from the pandemic period had 1 to 2 changes, and all strains in groups from post-pandemic period contained 2 to 3 changes (Table 3).

The large majority of these mutations were found at low frequency (63.9% to 98.6%) scattered throughout the yearcovered by our sampling, namely: T222A, T235 and S251 in C2; R307S, A308S and A308N in C2; N317D, S174V, S174F, G187R, and E230D in C1; A202T, D204Y, Q199K, and G188R in S1. However, other mutations at the five antigenic sites appeared in high proportion in the viral population. The C209T and K295E substitutions located at antigenic site C1, were found in 90.5% and 12.3% of strains respectively. In S1 63.5% of strains showed substitutions at residue 185, S185T (n = 126) or S185A (n = 7). In S1 we observed K160Q or K161H substitutions in 42.7% of strains. H1RDQ was found in 58.3% D222G, D222N and mixed viral populations D222G /D222N had a frequency of 55.3%.

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

Comparisons of phylogenetic groups and amino acid changes at the haemagglutinin gene of influenza A(H1N1)pdm09 viruses in 158 Brazilian patients with mild or severe/fatal clinical outcomes.

<table>
<thead>
<tr>
<th>Phylogenetic group (amino acid signature)</th>
<th>Number of mild cases (%)</th>
<th>Number of severe and fatal cases (%)</th>
<th>Polyphymorphisms at 375 sites of H5N1 obtained by pyrophosphorylation (number of sample with mutation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Un subgroup 1 pdm09 (H1A1)</td>
<td>3 (3%)</td>
<td>1 (3%)</td>
<td>0222 (2) and 0222, 0222, 0222 (1) and 0222, 0222 (1)</td>
</tr>
<tr>
<td>G6-pdm (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (85%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>C7-pdm (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>6 (83%)</td>
<td>0222 (2) and 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>G7 (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (83%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>G8 (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (83%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>C7 (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (83%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>G6 (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (83%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
<tr>
<td>C7 (H1A1-pdm09)</td>
<td>6 (16%)</td>
<td>8 (83%)</td>
<td>0222 (2), 0222, 0222 (4) and 0222, 0222 (4)</td>
</tr>
</tbody>
</table>

(Polyphymorphisms at multiple 375 sites of H5N1 were determined by noline et al (2011) protocol. The presence of these polyphymorphisms was significantly associated with severe fatal clinical outcomes (p-value 0.036), the statistical significance was calculated by Fisher’s exact test. |

4. Discussion

This study describes amino acid changes in the HA of 210 A(H1N1)pdm09 viruses circulating in Brazil during the pandemic period (April 2009 to August 2010) and in the following epidemic seasons (2010 to 2014). Phylogenetic analyses indicate that at least eight distinct genotypes are circulating 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, G3, G4, G5, G6, and G7 (H1A1-pdm09). We identified two of these early phylogenetic groups, namely G6 and G7, as well as four ungrouped pdm09 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 and G7-pdm09 co-circulated among Brazilian population confirming pre-existing data (Corsi et al., 2011; Oliveira et al., 2011; Samann et al., 2014). As observed worldwide, G7-pdm09 was the most common phylogenetic group detected in Brazil during the pandemic period (Helinnet al., 2009; Potdar et al., 2010). The global dominance of G7-pdm09 variant may be attributed to its initial foothold in New York City, which may have facilitated its rapid global spread due to its high international connectivity (Simon et al., 2011). The end of pandemic period was then defined on August, 2010 (World Health Organization 2010a) and from mid-2010 to early 2011, IJU 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-seasonal 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, 2012a, 2012b, 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, 2012). The increase of diversity was also observed in countries such as Turkey, India, Japan and Tunisia (Dahboh et al., 2013; Dapertet et al., 2012; El Messri et al., 2013; Goldstein et al., 2013). At least 10 new phylogenetic groups were detected circulating worldwide and they were numbered from group 2 to 7 (G2 to G7), since virus strain A/Cameroon/7/2000 was designated as G1 (European Centre for Disease Prevention and Control, 2011, 2012). 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 subgroup, 6A to 6C (European Centre for Disease Prevention and Control, 2011). Using the same approach, we describe a subgroup, with phylogenetic signature N200T, called G8. This mutation, detected among 2012 Brazilian samples seems to be observed mostly in IJU 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) was observed, however samples of 2014 epidemic season were detected only in the subgroup G6. According to data from Brazilian MoH, circulation of A(H1N1)pdm09 as a seasonal virus was more extensive during 2012 and 2013 epidemic seasons compared with the 2011 and 2014 epidemics (Secretary of Health Surveillance – Brazilian MoH, 2012, 2012a, 2012b, 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 during 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 3

<table>
<thead>
<tr>
<th>Phylogenetic Group</th>
<th>Number of sequences</th>
<th>Time of circulation in Brazil</th>
<th>Mean of amino acid distance to A/California/07/2009</th>
<th>Mean of predicted vaccine efficacy (%)</th>
<th>Number of changes in vaccine epitopes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ungrouped strains</td>
<td>4</td>
<td>Jun 2008 to Aug 2009</td>
<td>0.05</td>
<td>0.79</td>
<td>0</td>
</tr>
<tr>
<td>G6-pdm</td>
<td>17</td>
<td>Mar 2009 to Mar 2010</td>
<td>0.09</td>
<td>0.72</td>
<td>0</td>
</tr>
<tr>
<td>G3</td>
<td>44</td>
<td>Jun 2009 to Jun 2010</td>
<td>0.09</td>
<td>0.70</td>
<td>0</td>
</tr>
<tr>
<td>G2</td>
<td>22</td>
<td>Mar 2011 to Aug 2012</td>
<td>0.22</td>
<td>0.22</td>
<td>2</td>
</tr>
<tr>
<td>G1</td>
<td>2</td>
<td>May 2011 to Jun 2011</td>
<td>0.22</td>
<td>0.20</td>
<td>2</td>
</tr>
<tr>
<td>G2 (Q39F, R2SKD, D18, V208)</td>
<td>79</td>
<td>Mar 2013 to Jun 2014</td>
<td>0.23</td>
<td>0.23</td>
<td>2</td>
</tr>
<tr>
<td>G2 (Q39E, AD57)</td>
<td>2</td>
<td>May 2013 to Jun 2013</td>
<td>0.22</td>
<td>0.20</td>
<td>2</td>
</tr>
<tr>
<td>G2 (Q178)</td>
<td>33</td>
<td>Apr 2012 to Jan 2013</td>
<td>0.22</td>
<td>0.22</td>
<td>2</td>
</tr>
<tr>
<td>G2 (Q146, A197)</td>
<td>17</td>
<td>May 2012 to Jun 2013</td>
<td>0.22</td>
<td>0.22</td>
<td>2</td>
</tr>
</tbody>
</table>

(1) 95% confidence interval (2) p-value

Ledesma et al., 2012). It may suggest that the earlier epidemic in the North Hemisphere may precede and potentially seed the next pandemic 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 influenza strains from the vaccine and some of the amino acid substitutions occurred in antigenic sites (C11, C12, C13, and S14) of the HA molecule. Researchers and Suzuki, 2012). Although the genetic groups identified in this study showed specific changes in different epidemic cycles of HA, which contributed to the different strains' in-vitro efficacy, the results obtained by formal antigenic characterization in parallel with the variant viruses did not show a consistent marked change in antigenicity compared to vaccine strain A/California/07/2009. Therefore, new seasonal vaccine must be developed for 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 has 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/07/2009, but theoretically the vaccine efficacy has been decreased due to changes in antigenic sites of these strains. This study reinforces the importance of continued laboratory-based vaccine surveillance, which essential for the evaluation of new vaccine strains to the dominant circulating circulating variant and the effectiveness of the vaccine is likely to be.

Supplementary data in this article can be found online at http://dx.doi.org/10.1016/j.meegid.2015.08.007.

5. Conclusions

Fifteen years after the pandemic A/H1N1)pdm09: continuous circulation 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 has 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/07/2009, but theoretically the vaccine efficacy has been decreased due to changes in antigenic sites of these strains. This study reinforces the importance of continued laboratory-based vaccine surveillance, which essential for the evaluation of new vaccine strains to the dominant circulating circulating variant and the effectiveness of the vaccine is likely to be.

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