Evidence for an intranasal immune response to human respiratory syncytial virus infection in cynomolgus macaques

Clément Grandin,1,2,3 Marianne Lucas-Hourani,2,3 Marine Clavel,1 Fabrice Taborik,1 Astrid Vabret,4 Frédéric Tangy,2,3 Hugues Contamin1 and Pierre-Olivier Vidalain2,3

There is no large-scale therapy available against human respiratory syncytial virus (hRSV), a major pathogen responsible for acute respiratory diseases. Macaques represent an interesting animal model to evaluate potential treatments because of their genetic, anatomical and immunological proximity with humans. However, the parameters that influence hRSV growth and control in this model are still poorly understood. We have documented in the following study the influence of age as well as repeated infections on the virological, clinical and immunological parameters of this animal model. Following intranasal inoculation, hRSV replicated in the upper respiratory tract for less than 15 days with no clinical signs regardless of age. Interestingly, we observed the induction of a local immune response at the nasal mucosa as assessed by expression profiles of inflammatory and IFN-stimulated genes. Animals also developed specific antibodies and were immune to reinfection. Thus, we showed that even in infant macaques, intranasal hRSV infection induced both local and systemic immune responses to efficiently control the virus.

INTRODUCTION

Human respiratory syncytial virus (hRSV) belongs to the family Paramyxoviridae and the genus Pneumovirus, and can be divided into two separate groups (A and B). It is responsible for mild upper respiratory diseases with a high incidence in human population, but it can be associated with lower respiratory tract infections, with a bad prognosis in young infants, immunocompromised and elderly people (see Borchers et al., 2013; Collins & Melero, 2011 for reviews). Children reinfections with hRSV are extremely frequent and will occur until they develop a protective immunity. There is no vaccine available against hRSV infection and current therapies usually aim at limiting the inflammatory symptoms, without evidence of a positive impact on disease outcome. There is no vaccine available against hRSV infection and current therapies usually aim at limiting the inflammatory symptoms, without evidence of a positive impact on disease outcome. The FDA has approved nebulized ribavirin in the treatment of infants with severe bronchiolitis, but its effectiveness is still a matter of debate (Collins & Melero, 2011; Krilov, 2011). Palivizumab is a humanized mAb directed against the F protein of hRSV. It is approved to prevent hRSV infection during the epidemic season in high-risk infants and young children. Although clinical trials have demonstrated its efficacy, palivizumab prophylaxis remains limited essentially because of its current cost (Collins & Melero, 2011). Initial attempts to develop a hRSV vaccine with a formalin-inactivated virus did not induce any protective immunity and enhanced the disease symptoms in recipient infants (Borchers et al., 2013; Collins & Melero, 2011). Since then, several vaccine strategies, from live-attenuated strains to recombinant hRSV proteins, have been designed and tested with relative success in animal models. Only a few were evaluated in clinical trials and so far, none has shown sufficient safety and efficacy to be approved (Borchers et al., 2013; Collins & Melero, 2011).

Evaluation in animal models is usually considered an obligatory step in the development of anti-hRSV drugs or candidate vaccines. Humans are the only natural host for hRSV and animal models developed so far are only semipermissive to this virus (Bem et al., 2011; Borchers et al., 2013; Byrd & Prince, 1997). This includes mice, cotton rats, guinea pigs, sheep, chinchillas, African green monkeys, macaques and chimpanzees. These different models can be...
<table>
<thead>
<tr>
<th>Species</th>
<th>Age</th>
<th>Virus strain</th>
<th>Inoculum</th>
<th>Infection</th>
<th>Virus detection</th>
<th>Clinical signs/pathology</th>
<th>Reference</th>
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<tr>
<td><em>M. fascicularis</em></td>
<td>10–24 months</td>
<td>Macaque-adapted clinical isolate (RSV-A)</td>
<td>$10^6$ TCID$_{50}$</td>
<td>Intratracheal</td>
<td>BAL [quantitative reverse transcriptase (qRT)-PCR]</td>
<td>Bronchointerstitial pneumonia. Multinucleated syncytial cells and intracytoplasmic inclusion bodies</td>
<td>de Swart <em>et al.</em> (2002)</td>
</tr>
<tr>
<td><em>M. fascicularis</em></td>
<td>8–16 months</td>
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<td>$10^6$ TCID$_{50}$</td>
<td>Intratracheal</td>
<td>BAL (qRT-PCR)</td>
<td>Not reported</td>
<td>de Waal <em>et al.</em> (2004)</td>
</tr>
<tr>
<td><em>M. mulatta</em></td>
<td>Young adults</td>
<td>Macaque-adapted clinical isolate (RSV-A)</td>
<td>Not reported</td>
<td>Intranasal</td>
<td>Nasal and throat swabs, BAL (qRT-PCR)</td>
<td>None</td>
<td>Grunwald <em>et al.</em> (2014)</td>
</tr>
<tr>
<td><em>M. mulatta</em></td>
<td>Young adults</td>
<td>RSV A2 or RSV B1</td>
<td>$10^5$ p.f.u.</td>
<td>Intratracheal and intranasal</td>
<td>Nasal swabs and tracheal lavages (titration)</td>
<td>Not reported</td>
<td>Schmidt <em>et al.</em> (2002)</td>
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<td><em>M. mulatta</em></td>
<td>1.5–5.5 months</td>
<td>Clinical isolate</td>
<td>$5 \times 10^6$ TCID$_{50}$</td>
<td>Aerosolization</td>
<td>Nasopharyngeal washes (immunofluorescence)</td>
<td>Bronchiolar and interstitial pneumonia. Foamy alveolar macrophages. Lymphocytic, histiocytic and eosinophilic infiltrates in the septa</td>
<td>McArthur-Vaughan &amp; Gershwin (2002)</td>
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<tr>
<td><em>M. mulatta</em></td>
<td>3 months</td>
<td>Clinical isolate</td>
<td>$10^7$ TCID$_{50}$</td>
<td>Aerosolization</td>
<td>Lung after necropsy [reverse transcriptase (RT)-PCR]</td>
<td>Mild pneumonia. Lymphocytic and histiocytic infiltrates in the septa</td>
<td>Vaughan <em>et al.</em> (2005)</td>
</tr>
<tr>
<td><em>M. mulatta</em></td>
<td>Young adults</td>
<td>Macaque-adapted RSV Long</td>
<td>$10^{5.7}$ TCID$_{50}$</td>
<td>Intranasal</td>
<td>Nasal and throat swabs, BAL (titration)</td>
<td>Not reported</td>
<td>Weltzin <em>et al.</em> (1996)</td>
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<tr>
<td><em>M. mulatta</em></td>
<td>1 week (infants)</td>
<td>RSV A2</td>
<td>$0.5 \times 10^5$ p.f.u.</td>
<td>Intranasal</td>
<td>Nasal and throat swabs (titration)</td>
<td>None</td>
<td>Belshe <em>et al.</em> (1977)</td>
</tr>
<tr>
<td><em>M. radiata</em></td>
<td>Infants and juveniles</td>
<td>RSV Long</td>
<td>$5 \times 10^6$ p.f.u.</td>
<td>Intrabronchial</td>
<td>BAL (titration) and lungs after necropsy (in situ hybridization)</td>
<td>None</td>
<td>Ponnuraj <em>et al.</em> (2001)</td>
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used to evaluate vaccine and drug candidates, but they only recapitulate some aspects of human immunopathology. Cognate host–pneumovirus models include bovine respiratory syncytial virus infection in cattle and mouse pneumovirus infection in mice. Non-human primates are often preferred at the preclinical stage because of their genetic, anatomical and immunological proximity with humans, and macaques are most frequently used because they are relatively easy to breed. Experimental hRSV infections of three macaque species have been previously described, including rhesus (Macaca mulatta) (Belshe et al., 1977; Grunwald et al., 2014; McArthur-Vaughan & Gershwin, 2002; Schmidt et al., 2002; Vaughan et al., 2005; Weltzin et al., 1996), bonnet (Macaca radiata) (Babu et al., 2001; Simes et al., 1999) and cynomolgus monkeys (Macaca fascicularis) (de Swart et al., 2002; de Waal et al., 2004a, b). Experimental designs and major results are summarized in Table 1. Infections were performed with clinical isolates or laboratory strains (strain Long) of hRSV that were essentially from group A. Viruses were macaque-adapted prior to administration in some experimental designs. Inocula usually ranging from $10^5$ to $10^7$ TCID$_{50}$ were administered by the intranasal, intratracheal or intrabronchial route, or delivered by aerosolization. Infants or young adults were infected and viral replication was determined by assessing nasal and throat swabs as well as bronchoalveolar lavages (BALs). All animals showed some level of viral replication with a peak between days 5 and 9, and resolved the infection. Lung inflammation associated with cellular infiltrates was also observed, except in two reports where animals were infected by the intranasal route (Belshe et al., 1977; Grunwald et al., 2014). These reports thus illustrated that the macaque is an interesting model for preclinical evaluation of vaccines or treatments against hRSV. However, parameters that influence hRSV growth and control in this model, including the age, the viral strain, the administration route as well as the immunological response require further documentation. Based on these previous reports, our goal was to study in cynomolgus macaques the influence of age and repeated infections on the virological, clinical and immunological parameters.

**RESULTS**

**Comparison of hRSV infection in infant, 1-year-old and adult cynomolgus monkeys**

Four infants (4 months; IDs I34–I37), three juvenile animals (1 year old; IDs Y31–Y33), and three adult animals (7–20 years old; IDs A38–A40) were infected with $10^5$ TCID$_{50}$ of hRSV. Infections were conducted with the widely used hRSV-A laboratory strain because so far, the use of clinical isolates has not shown any obvious benefit in this model. The virus was administered drop-wise by the intranasal route, which is less traumatic and more physiologically compared with an intratracheal administration. One additional juvenile animal was mock-treated and used as a negative control (Y30). Clinical examination was performed and biological samples collected according to the experimental timeline described in Fig. 1. Animals Y30 and Y31 were sacrificed at day 9, for necropsy and tissue analysis.

Infected animals did not show any clinical signs except occasional sneezing. No significant variations in body weight, temperature, respiratory rate and heart rate were detected. Blood cell counts did not reveal significant variations in any age group. An increase in eosinophil counts was observed in blood samples and BALs from infected 1-year-old animals, but this trend was not observed in other age groups and could be due to BAL collection (data not shown).

To estimate hRSV replication in the upper respiratory tract, viral RNA copies in nasal swabs were quantified by quantitative reverse transcriptase PCR and normalized relative to the total cell count in each specimen. The initial inoculum was clearly detected at day 1 post-infection (Fig. 2a–f). The mean of the results showed that viral RNA copies decreased from day 1 to day 3 and subsequently increased to reach a peak between day 5 and day 9 (Fig. 2b, d, f). Interestingly, the numbers of viral RNA copies detected in each nostril of an infected animal were sometimes found to be very different, suggesting that both should be tested to properly assess the viral growth in a given animal (Fig. 2a, c, e). Finally, some infectious virus could be recovered from nasal swabs of infected animals despite freezing and long-term storage of these specimens at $-80$ °C (data not shown).

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**Fig. 1.** Timeline of infection and samples collection. Infant, 1-year-old and adult animals were infected intranasally with $10^5$ TCID$_{50}$ hRSV. Nasal swabs and blood samples were collected from all animals at indicated time points. BALs were only collected on 1-year-old animals.
Fig. 2. Viral RNA copies per $10^3$ cells detected in nasal swab specimens from infected infants (a, b; showing both first and second infection), 1-year-old animals (c, d), and adults (e, f). hRSV RNA copies in nasal swab specimens were quantified by qRT-PCR and normalized relative to cell count in each sample (also estimated by qRT-PCR). Results in (a), (c) and (e) correspond to hRSV RNA copies detected in each animal and show separate data obtained from the left and right nostrils (solid and dotted lines, respectively). Results in (b), (d) and (f) correspond to means and SD for each age group. First, log values of viral RNA copies from left and right nostrils of each animal were averaged. Then, the mean and SD were calculated for each time point. Since animal I36 was an outlier in the second infection, data points were excluded to calculate mean values and SD as shown in (b; right panel).
Viral RNA copies were also quantified in BALs from 1-year-old animals to determine if the virus had reached the lower respiratory tract. Viral RNA copies were only detected in one of the three infected animals (Y33) and signal was 1500 times lower compared with nasal swabs from the same animal (Fig. 3). Viral RNA copies detected in the lower respiratory tract corresponded either to nasal discharges or background replication in the lungs. In any case, this showed that hRSV replication in this model is mostly – if not exclusively – occurring in the upper respiratory tract. Supportive of this statement, when sacrificed at day 9, the histopathology showed no obvious sign of viral replication or inflammation in the upper and mid-respiratory tract in animal Y31 compared with the non-infected control Y30 (data not shown).

**hRSV-infected animals were protected from reinfection**

Next, we checked if the first infection had induced an adaptive immune response in infant monkeys and whether this would either prevent a second infection or exacerbate symptoms. At day 94 after the first infection, all four animals were challenged again and monitored for 15 days. No clinical signs were observed throughout this period. The viral inoculum was detected at day 1 post-infection, but viral RNA copies in nasal swabs quickly declined in all animals except I36 that showed hRSV replication in the left nostril (Fig. 2a, b). Altogether, this illustrated the induction of a protective immune response in all animals but I36 following the first infection.

This conclusion was also supported by measures of anti-hRSV antibodies in serum samples before and after the first and the second infection. As shown in Table 2, anti-hRSV antibodies were detected in all animals at days 28 and 92 post-infection. However, titres were clearly lower in I36.

**Expression of inflammatory and IFN-inducible genes in the nasal mucosa**

We next determined, in the upper respiratory tract of infected macaques, the expression profile of 12 inflammatory cytokines and IFN-inducible genes that were previously found induced in patients or hRSV-infected airway epithelial cells (Bermejo-Martin et al., 2007; Choi et al., 2010).

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**Table 2. Serum anti-hRSV antibodies detected by ELISA and seroneutralization**

<table>
<thead>
<tr>
<th>Monkey no.</th>
<th>Days post-infection</th>
<th>hRSV IgG/M*</th>
<th>Neutralizing antibody†</th>
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<tr>
<td>I34</td>
<td>−2</td>
<td>&lt;200</td>
<td>&lt;16</td>
</tr>
<tr>
<td>I35</td>
<td>−2</td>
<td>&lt;200</td>
<td>&lt;16</td>
</tr>
<tr>
<td>I36</td>
<td>−2</td>
<td>&lt;200</td>
<td>&lt;16</td>
</tr>
<tr>
<td>I37</td>
<td>28</td>
<td>1124</td>
<td>64</td>
</tr>
<tr>
<td>I34</td>
<td>28</td>
<td>1372</td>
<td>32</td>
</tr>
<tr>
<td>I36</td>
<td>28</td>
<td>551</td>
<td>ND</td>
</tr>
<tr>
<td>I34</td>
<td>92</td>
<td>3019</td>
<td>32</td>
</tr>
<tr>
<td>I35</td>
<td>92</td>
<td>1971</td>
<td>32</td>
</tr>
<tr>
<td>I36</td>
<td>92</td>
<td>616</td>
<td>16</td>
</tr>
<tr>
<td>I37</td>
<td>92</td>
<td>2943</td>
<td>32</td>
</tr>
<tr>
<td>I34</td>
<td>285</td>
<td>2785</td>
<td>32</td>
</tr>
<tr>
<td>I35</td>
<td>285</td>
<td>1124</td>
<td>64</td>
</tr>
<tr>
<td>I36</td>
<td>285</td>
<td>2696</td>
<td>32</td>
</tr>
<tr>
<td>I37</td>
<td>285</td>
<td>1243</td>
<td>128</td>
</tr>
</tbody>
</table>

ND, Not determined.

*End-point dilution titre determined by ELISA.
†End-point dilution titre determined by seroneutralization.

This suggested the induction of a poor memory response following the first infection and probably explains I36 susceptibility to reinfection at day 94. More than 6 months after the second infection, antibody titres were relatively high and quite similar in all animals including I36 (Table 2; see day 285 after the first infection). Serum samples were also tested for the presence of anti-hRSV neutralizing antibodies. Results clearly correlated with anti-hRSV antibody titres obtained by ELISA. In conclusion, this demonstrated the induction of a long-lasting and protective memory response in this model, even when applied to infant macaques. In agreement with the observations made on infant animals, anti-hRSV antibodies were also detectable at day 37 post-infection in all three adults (Table S1, available in the online Supplementary Material). In contrast, only intermediate seroconversions were observed in 1-year-old animals at days 9 or 15, which corresponds to the time of their sacrifice, suggesting that the induction of a robust antibody response requires more than two weeks (Table S1).
Nasal swab specimens from infant animals were analysed throughout the first and the second infection. Moreover, we also determined, in non-infected animals (between the two infections at day 68), the influence of repeated daily swabbing on gene expression.

Expression profiles of CXCL10, CXCL11, TNF-α and IFN-β were inconsistent from one animal to another and could not be interpreted (data not shown). The eight remaining genes displayed specific activation patterns that could be clustered into at least three groups. The first set included IFI35 and CCL5; this set consisted of genes that were never induced in the first, second or mock infection of infant animals (Figs 4a and 5a). One-year-old and adult animals showed consistent profiles for IFI35 (Fig. 5a, b) and CCL5 (Fig. 5a, b), although a significant induction of CCL5 was observed in one of the young animals (Fig. 5a, Y32). Thus, IFI35 and CCL5 genes were essentially unresponsive to hRSV infection in the macaque model described herein, despite evidences of their induction in infected airway epithelial cells or patients (Culley et al., 2006; Harrison et al., 1999; Ioannidis et al., 2012; Murai et al., 2007).

IL-8 and CSF1 were induced in hRSV-infected infant macaques (Figs 4b and S1b). However, these two genes were also induced by nasal swabbing alone, even at earliest time points. Thus, IL-8 and CSF-1 induction rather reflected a local inflammation induced by mechanical abrasion of the nasal mucosa. Consequently, results obtained in 1 year olds and adults could not be interpreted (Fig. 5c, d and S2c, d). Altogether, this strongly stresses the need for appropriate mock controls when performing such experiments.

With this precaution in mind, we successfully identified a set of genes that was clearly activated by hRSV infection. First, Mx2 was strongly induced in all infant animals following the first infection (Fig. 4c). Repeated swabbing had a limited impact, with some induction only detected at the latest time points. No induction of Mx2 could be detected after the second infection, except in the left nostril of I36 at day 7, which paralleled the viral load detected in this specimen. Thus, Mx2 induction in nasal swab specimens essentially correlated with hRSV replication in infant animals and this demonstrated the induction of a local immune response in these animals. Consistent results were obtained in 1-year-old and adult macaques, although Mx2 induction was much less pronounced in the latter group (Fig. 5e, f). OASL and IL-1z transcription profiles were relatively similar. However, the repeated swabbing alone was found to have more impact on the expression of these two genes at late time points thus complicating the interpretation (Figs. S1c, d and S2e–h). IL-6 expression was also induced in infant animals following the first infection and not by nasal swabbing alone. Moreover, only a limited induction was observed after the second infection (Fig. 4d). Thus, the IL-6 expression level correlated with viral replication although this induction was not detected in older animals (Fig. 5g, h), suggesting that it was age-dependent.

**DISCUSSION**

We have investigated the influence of age and repeated hRSV infections on virological and immunological parameters in macaques. The virus was administered by the intranasal route, which leads to a sustained replication in the nasal cavity in agreement with previous publications (Grunwald et al., 2014; Weltzin et al., 1996), but with a low level of viral shedding in the lungs when compared with intratracheal inoculation or aerosolization (Babu et al., 1998; de Swart et al., 2002; de Waal et al., 2004a, b; McArthur-Vaughan & Gershwin, 2002; Ponnuraj et al., 2001; Simes et al., 1999; Vaughan et al., 2005). Interestingly, we found important differences between the two compartments of the nasal cavity, both in terms of hRSV RNA copies and immune response. This suggests that a better detection of this pathogen in clinical samples for diagnostic could be achieved by systematically swabbing both nostrils, using nylon flocked swabs as performed herein (DeBye et al., 2012; Heikkinen et al., 2002).

One of our main objectives was to compare the outcome of this infection in different age groups. Indeed, first experimental hRSV infections in macaques were performed in 1977 on newborns (Belshe et al., 1977). Since then, other studies have used infant animals without a strong rational aside from the higher susceptibility of human infants to hRSV. In fact, we found no significant differences between infants, young animals and adults in terms of susceptibility to infection or physiopathology. Nevertheless, expression levels of Mx2, OASL, IL-1z or IL-6 were higher in infants compared with young animals and adults, suggesting that the intranasal immune response is age-dependent without consequences on clinical outcomes.

The induction of a poor immune memory response is usually presented as the main reason for human reinfection by hRSV and is a major obstacle to develop vaccine. A previous report established that after two intratracheal infections, bonnet monkeys are protected from reinfection (Ponnuraj et al., 2001). Here, we showed that 3 months after a single intranasal infection, infant macaques benefit from a good protection against reinfection. The duration of this immunity remains to be investigated, but the level of the specific antibodies in the blood samples remained stable for at least 6 months after reinfection. Therefore, macaques are clearly useful to evaluate candidate hRSV vaccines, but this is probably not a stringent model and vaccines performing well in macaques could be inefficient once transferred to human.

Finally, we documented for the first time to our knowledge the inflammatory and innate immune response triggered by hRSV in the intranasal cavity of infected macaques. The gene expression profiles turned out to be altered by the actual act of repeated nasal swabbing, which induced some local inflammation and thus generated artefacts. Notably, hRSV infection actually interfered with this phenomenon and either partially (first infection), or completely (second infection) suppressed the induction of inflammatory genes like IL-8 by repeated nasal swabbing. It is conceivable that
Fig. 4. Relative expression levels of IFI35 (a), IL-8 (b), Mx2 (c) and IL-6 (d) mRNAs in nasal swab specimens from infected infants during first, mock and second infection. Expression levels of IFI35, IL-8, Mx2 and IL-6 were determined by qRT-PCR. For each one of these genes, Ct values were normalized to the average of Ct values of four housekeeping genes in the same sample (18S, GAPDH, GUSB and ACTB) and expressed as fold changes relative to the first nasal swab specimen of each of the three procedures (ΔΔCt method).
Fig. 5. Relative expression levels of IFI35, IL-8, Mx2 and IL-6 mRNAs in nasal swab specimens from 1-year-old (a, c, e and g, respectively) and adult (b, d, f and h) animals after hRSV infection. Expression levels of IFI35, IL-8, Mx2 and IL-6 were determined by qRT-PCR. For each one of these genes, Ct values were normalized to the average of Ct values of four housekeeping genes in the same sample (18S, GAPDH, GUSB and ACTB) and expressed as fold changes relative to expression levels in the first nasal swab specimen collected before infection (ΔΔCt method).
immune response components induced by hRSV infection or virulence factors encoded by the virus itself, are thus interfering with the inflammation induced by repeated swabbing. When infecting macaques for the second time, anti-hRSV antibodies could also suppress inflammation by the engagement of membrane inhibitory receptors like CD32b/FcγRIIB (Smith & Clatworthy, 2010). In particular, isoforms 1 and 2 of FcγRIIB exhibit an immunoreceptor tyrosine-based inhibitory motif within their cytoplasmic tails. As a consequence, their engagement by immune complexes efficiently inhibits the release of pro-inflammatory cytokines and the activation of immune effector cells like macrophages, dendritic cells and granulocytes.

Despite these technical difficulties, we established that a local immune response is induced at the nasal mucosa, as assessed by Mx2, OASL, IL-1x and IL-6 profiles. Most importantly, the expression of type I IFN-stimulated genes such as Mx2 and OASL suggests that an innate immune component is involved in agreement with recent reports addressing this point in human patients (Fjaerli et al., 2006; Scagnolari et al., 2007). Most interestingly, Scagnolari et al. (2007) have established an inverse correlation between expression levels of IFN-stimulated genes and clinical scores, suggesting that a poor innate immune response is associated with a severe disease. It is possible that hRSV replicates at relatively low levels in macaques and other primates when compared to chimpanzees and human because the virus is unable to properly control the innate immune response. Whether this represents a host species barrier for hRSV needs to be investigated.

**METHODS**

**Statement of ethics.** All procedures were conducted in accordance with the French regulation relative to the protection of animals used for scientific purposes and with European guidelines (ETS 123 from the Council of Europe). Procedures were reviewed and approved by an external Ethics Committee under number 1279 (VetAgro Sup; Marcy l’Etoile, France) and by the Cynbiose Animal Welfare Body. The animal husbandry programme in Cynbiose was designed to minimize pain and distress and included group housing, ad libitum access to water, appropriate amounts of food and environmental enrichment such as toys, fresh fruits and foraging equipment. Animals were under constant supervision of trained technicians and veterinarians.

**Virus stock production.** Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM; Life Technologies) containing 10% FCS, penicillin and streptomycin at 37°C and 5% CO₂. hRSV-A Long from ATCC was amplified first on human Hep-2 cells, and then passaged once on LLC-MK2 epithelial kidney cells from macaque to eliminate human cellular factors. Virus stock was titrated on LLC-MK2 cells by TCID₅₀. Because cytopathic effects on LLC-MK2 cells are limited, infection in titration plates was determined by anti-hRSV immunostaining (mAb8599; Millipore).

**Animals and virus inoculations.** Eight cynomolgus monkeys (M. fascicularis) of Mauritian origin were purchased from Bioprimg and housed in the Cynbiose animal facility for at least 2 weeks before experiments were started. Purchased animals were four 1-year-old males and four pregnant adult females. Females delivered 2 months after their arrival at Cynbiose, and young infants were housed in groups with their mothers. Animals were housed in biosafety level 2 facilities during experimental phases. Infants were infected for the first time at 4 months of age, while they were still housed with their mother. Infection of the adult animals and the second infection of infant animals both took place after weaning. Monkeys were infected intranasally under intramuscular ketamine sedation (10 mg ketamine kg⁻¹) with 10⁶ TCID₅₀ of hRSV diluted in 200 μl PBS (pipetting 100 μl drop-wise into each nostril). Their head was rocked back and forth and monkeys were maintained lying on their back for 10 min before being returned to their enclosure. Clinical examination and pulmonary auscultation were performed by a trained veterinarian. All animals were seronegative before infection as assessed by ELISA and seroneutralization assay, except animal A38 that showed some signal by ELISA just at the threshold level. This might reflect a mild contamination with hRSV when this adult female was in close contact with her infected infant, but this cannot be asserted as the signal was extremely weak. Viral replication and gene expression profiles in A38 were comparable to the other animals from the same group of age, suggesting that it had no impact on the infection.

**Blood samples and haematology.** Blood samples were collected in serum tubes and EDTA-coated tubes. Serum was separated by centrifugation and stored at −80°C. Blood samples were analysed for haematological parameters with a Sysmex XT2000i Vet automatic analyser (Sysmex SAS).

**Nasal swabs and BALs.** Nasal specimens were collected daily from conscious animals in each nostril with paediatric, flocked swabs (516SC01; Copan). Swabs were placed in UTM tubes containing viral transport medium (350C; Copan) and stored at −80°C.

BAL samples were collected every 3 days in 1-year-old animals under general anaesthesia consisting in intramuscular ketamine (10 mg ketamine kg⁻¹) and midazolam (1 mg midazolam kg⁻¹). The trachea were infused with a solution of 5 ml isotonic sodium chloride kg⁻¹, which was subsequently recovered with a flexible catheter. An aliquot of BAL samples was cytospinned at 182 relative centrifugal force for 10 min and the differential cell count was performed by a certified veterinary clinical pathologist. Remaining BAL samples were pelleted and frozen as dry pellets at −80°C.

**qRT-PCR analysis of hRSV replication and cellular gene expression.** Total RNA from nasal swabs transport medium and BAL pellets was isolated with the RNAeasy Mini kit (74104; Qiagen) and analyzed on a QIACube robotic workstation (Qiagen) according to the manufacturer’s protocol. Following elution, RNA yields were evaluated using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific). Extracted RNA was stored at −80°C.

RNAS corresponding to hRSV were detected in biological samples by one-step real-time PCR using Applied Biosystems TaqMan RNA-to-C₅T kit (4392938; Life Technologies). Forward and reverse primers specific for hRSV-N correspond to previously published sequences (van de Pol et al., 2010), but the TaqMan detection probe was shorter and fused to MGB for increased DNA binding (5’-CACCATGCAACGGAG-MGB). The number of hRSV-N RNA copies in each biological sample was determined using a reference dilution curve of viral RNA molecules [obtained by in vitro transcription from a plasmid containing hRSV-N DNA sequence (AY911262.1)]. Finally, results were normalized relative to cell count as determined by qRT-PCR using the CELL Control r-gene kit (71-106; Argene) and expressed as a number of viral RNA copies per 10⁶ cells. We did not observe any significant variation in total cell counts during hRSV infection.

A two-step qRT-PCR was performed to measure transcription levels for 12 genes of interest on TaqMan Array Plates (Life Technologies, primer references are indicated): CXCL10 (Rh02788358_m1), OASL (Rh02789783_m1), IL-6 (Rh02789832_m1), IL-8 (Rh02789871_m1),...
CCL5 (Rh02787153_m1), CXCL11 (Rh02621763_m1), TNF (Rh02789783_m1), CSF1 (Rh02621778_m1), IL-1 (Rh02789772_m1), IFI35 (Rh01113891_m1), Mx2 (Rh08242285_m1) and IFN-βi (Rh03648734_s1). Expression levels of four housekeeping genes, including 18S (Hs99999901_s1), GAPDH (Rh02621745_g1), ACTB (Rh03043379_gH), and GUSB (Rh02788764_m1) were also determined. Starting from 88 ng total RNA, cDNA synthesis was achieved using the SuperScript VILO™ cDNA Synthesis kit (11754-250 Life Technologies). Relative quantification PCRs were performed on 5 ng cDNA using the TaqMan Fast Universal PCR Master Mix (4364103 Life Technologies) on an Applied Biosystems StepOnePlus Real-Time PCR machine (4376600 Life Technologies).

Gene expression levels were expressed as fold changes (relative quantification) relative to control specimens collected one or two days before infection using the ΔΔCt method (Schmittgen & Livak, 2008). Because Ct values of the four housekeeping genes (18S, GAPDH, GUSB and ACTB) were consistent in each sample, Ct values of the four housekeeping genes were averaged. This average value is less sensitive to small variations in the expression of individual housekeeping genes and provides a better reference to normalize total RNA levels between samples (Arai et al., 2010). Thus, ΔCt corresponds to the Ct value of a gene of interest minus the average of Ct values of the four housekeeping genes. The ΔΔCt value was then calculated as the ΔCt value at a specific time point minus the ΔCt value one or two days before the corresponding infection. Two to the power −ΔΔCt corresponds to gene expression level at a specific time point expressed as a fold change relative to pre-infection.

**hRSV-specific antibody response.** Antibody titres were determined by ELISA (abi108765; Abcam), which was adapted by adding a peroxidase-conjugated anti-monkey IgG, IgA or IgM antibody from goat (617-103-130; Rockland Immunochemicals) to the kit solution. Each serum sample was serially diluted from 1:100 to 1:3200 and the titration end point was calculated from the best fitting quadratic equation at a 0.09 cut-off. The ELISA was benchmarked using serum samples from a cohort of ten animals freshly imported by Cynbiose as part of a separate research programme, and as such were supposedly naive to hRSV. From this training set, it was determined that serum samples should be considered negative when titration end points were <200.

To quantify neutralizing antibodies, sera were decanted by centrifuging at 56 °C during 30 min and serially diluted from 1:8 to 1:1024 in DMEM. In 96-well microplates, 50 μl of each serum dilution were mixed with an equal volume of hRSV at 100 TCID50 ml⁻¹ and then incubated for 1 h at 4 °C. Finally, 100 μl HEP-2 cell suspension at 10⁵ cells ml⁻¹ were added to each well. Microplates were incubated at 37 °C and cytopathic effects were determined with an inverted microscope. Neutralizing titres correspond to the last serum dilutions that entirely blocked hRSV cytopathic effects in the corresponding culture wells. Experiments were performed in triplicate and results expressed as average titres.

**ACKNOWLEDGEMENTS**

This work was supported by the National Research Agency (ANR-RPIB, Program STING 2.0), Lyonbiopôle, Institut Pasteur and the Centre National de la Recherche Scientifique. C.G. was supported by a CIFRE fellowship (Conventions Industrielles de Formation par la Recherche, Association Nationale de la Recherche et de la Technologie). We would like to thank Dr Yves Janin for proofreading this manuscript.

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