Role of p53/NF-κB functional balance in respiratory syncytial virus-induced inflammation response

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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 abrogation mechanisms 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 the expression of several proinflammatory cytokines and chemokines.

INTRODUCTION

Respiratory syncytial virus (RSV), discovered more than 60 years ago [1, 2], is one of the main causes of respiratory tract infection 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 up-regulate the expression of host genes involved in the antiviral and cell-mediated immune responses, such as gene coding for IFNs (IFN-alpha/beta) or several cytokines/chemokines [12–14]. RSV non-structural proteins, NS1 and NS2, are known to counteract IFN induction and signalling [15–23]. Interestingly, while the nuclear factor-kappaB (NF-κB)/REL family of transcription factors plays a central role in coordinating the expression of a wide variety of genes that control immune responses [24], several studies have highlighted the multiple levels of interplay between the NF-κB pathway and RSV during the time course of infection [25–31]. In that regard, NS1 and NS2 have been shown to indirectly contribute to the RSV-induced activation of NF-κB [17, 31, 32].

Another key player in the regulation of inflammation and immune responses is the cellular gatekeeper p53 (reviewed in [33]). The interplay between RSV and this transcription factor has only been reported in a limited number of studies [34–36]. Groskreutz and colleagues have shown that RSV induces the down-regulation of p53 during the time course of infection, through an Akt-dependent activation of its negative regulator Mdm2, with a consequent impact on apoptosis and survival of airway epithelial cells [34]. However, the underlying abrogation mechanisms of p53 activity during the time course of...
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 underline 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-κB 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 first investigated the impact of RSV infection on endogenous p53, by performing mock or RSV (Long strain) infections in A549 human lung epithelial cells, using different infection parameters to explore early and late stages of infection (Fig. 1). At an m.o.i. of 4, we measured an increase of p53 levels at 6 h post-infection (h p.i.) (Fig. 1a, left panel), with more than 1.8-fold increase of p53 relative protein levels (RPL) compared to the mock (Fig. 1b, left panel). In contrast, a slight but significant decrease on p53 RPL compared to the mock was observed at 12 h p.i. (Fig. 1b, left panel). Moreover, a marked reduction of p53 RPL at late stages of infection (48 h p.i.) was also observed using different infection parameters (m.o.i. of 1), with a more than 60% decrease in RSV-infected cells compared to the mock (Fig. 1a, right panel, P<0.001). To further investigate the impact of RSV on the endogenous p53 expression, we measured p53 mRNA levels by real-time quantitative PCR (RT-qPCR) in the same experimental conditions (Fig. 1c). Besides a moderate decrease at 6 h p.i., p53 mRNA levels in RSV-infected cells remained comparable to those measured in the mock control, regardless of the m.o.i. and sampling time conditions (m.o.i. 4–12 h p.i., m.o.i. 1, 24/48 h p.i., 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 the mock. This decrease of p53 transcriptional activity in infected cells was notably marked at 48 h p.i., with a more than 50% loss of luciferase activity compared to the mock (Fig. 1d, right panel), in correlation with a marked decrease of protein levels at the same time points (Fig. 1a, b, right panel). At 6 and 24 h p.i., the increase of p53 protein levels in RSV-infected cells (Fig. 1a, b) was not correlated with corresponding p53 mRNA levels (Fig. 1c) and transcriptional activity (Fig. 1d), suggesting a transitory stabilization and impairment of p53 activity. 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 an m.o.i. of 1 for 24 or 48 h, and then analysed p53 stability by monitoring protein levels over a 50 min period, after treatment with 50 µg ml⁻¹ of cycloheximide (CHX) protein synthesis inhibitor (Fig. 2a). As expected, the estimated p53 half-life in the mock was 20 min, in agreement with previous works using a similar cellular model and experimental conditions [37]. 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 h p.i., p53 levels were undetectable by Western blot (Fig. 2a). We then further investigated this strong destabilization of p53 at 48 h p.i. in the 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, 38]. 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 fluorescenceconfocal microscopy. After 24 h post-transfection, there was no clear impact of NS1 or NS2 expression on p53 staining and subcellular localization, which is mostly nuclear (Fig. 3a). The observed localization of NS1 and NS2 were in good agreement with previously published studies [21, 39]. In parallel, A549 cells were also transfected with either an empty plasmid or increasing amounts of plasmids expressing RSV NS1-eGFP (Fig. 3b) or NS2-eGFP (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-transfected 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 the mock (P<0.05 and <0.01, 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 the mock, for 0.2 and 2 µg of transfected NS2, respectively, and without strong impact on p53 mRNA expression (Fig. 3c). A similar impact on p53 transcriptional activity was also observed in the context of NS1/NS2 co-transfection (Fig. 3d). To further understand the contribution of NS1/NS2 to the modulation of p53 activity, we transfected A549

Fig. 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 an m.o.i of 4 or 1, and analysed at 6–12 h.p.i. (left panels) or 24–48 h.p.i. (right panels), respectively. (a) Cell lysates were analysed by Western blot for the expression of p53. Ku80 was used as a loading control. (b) p53 relative protein levels (p53 RPL) were measured by densitometry. (c) p53 mRNA expression was measured by RT-qPCR. (d) p53 transcriptional activity was monitored using a specific luciferase reporter assay. Relative luciferase units (RLU) reflect global transcriptional activity compared to the mock-infected control. All results were calculated from data from three independent experiments. *, ** and *** stand for P-values <0.05, 0.01 and 0.001, respectively.
cells with 2 µg of empty plasmid or NS1/NS2-expressing plasmids, and we assessed p53 stability as described before (Fig. 3e). 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. 3e). 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. 3e). 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 down-regulates 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. We hypothesized that a decrease of p53 expression before RSV infection would provide a favourable cellular environment for infection. A549 cells were first transfected with either a non-specific siRNA (si-Ctrl) or a pool of siRNAs targeting p53. Then, 48 h post-transfection, cells were infected with RSV at an m.o.i. of 1 and viral supernatants and cell extracts were harvested at 48 h p.i. Western blots confirmed that p53 levels were reduced in cells transfected by si-p53 compared to si-Ctrl (Fig. 4a). As expected, we observed that RSV genome copy numbers were significantly increased (twofold increase, *P* < 0.001) in supernatants of si-p53 transfected cells, compared to those of si-Ctrl transfected cells. This increase was also confirmed by infectious titres (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 p53. H1299 cells were transfected with either an empty plasmid or a plasmid expressing p53. Then, 48 h after transfection, cells were infected with RSV (m.o.i. of 1) and the impact of transient expression on viral production was assessed at 48 h p.i. The adequate transient expression of p53 was confirmed by Western blot. In these conditions, viral production was significantly lower in cells transfected with p53, with a more than 2.5-fold decrease on genome copy numbers (*P* < 0.001), and a 2-log10 reduction on infectious viral titres (Fig. 4b). In parallel, we advantageously used Nutlin-3, a small molecule known to bind the Mdm2 E3-ubiquitin ligase, therefore inducing the stabilization and activation of endogenous p53 [40]. A549 cells were
Fig. 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 immuno-fluorescence confocal microscopy. The expression of NS1 or NS2 is represented in green (eGFP), and p53 in red. Nuclei were counter-stained with DAPI. Scale bar=25 µM. (b) A549 cells were transfected with either an empty plasmid (pEGFPC1) or two different amounts of plasmid expressing RSV NS1-eGFP (0.2 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 plasmid expressing RSV NS2-eGFP. (d) A549 cells were transfected with either an empty plasmid or plasmids expressing RSV NS1-eGFP or RSV NS2-eGFP alone or in combination (1 µg). p53 global transcriptional activity was measured 48 h post-transfection with luciferase reporter assay (RLU). (e) Stability assay in the presence of NS1 or NS2. A549 cells were transfected with either an empty plasmid (Empty) or plasmids expressing RSV NS1-GFP or RSV NS2-GFP (2 µg). At 48 h post-transfection, p53 protein stability was assessed by monitoring relative protein levels (RPL) of p53 during a 50 min period, after treatment with 50 µg ml\(^{-1}\) cycloheximide (CHX). Mean values±SD from three independent experiments are presented. All results were calculated from data from three independent experiments. *, **, *** and **** stand for P-values <0.05, 0.01, 0.001 and 0.0001, respectively.
mock-infected or infected with RSV (m.o.i. of 1), in the presence of either Nutlin-3 (10 µM) or control DMSO (Ctrl). Treatment with Nutlin-3 significantly decreased viral production compared to the control, with an almost three-fold decrease on genome copy number ($P < 0.001$) and a 3-log10 reduction on viral titres (Fig. 4c). These results were consistent with our observations using siRNA and transient expression, suggesting an antiviral contribution of p53 in the context of RSV infection.

**RSV infection modulates the p53/NF-κB balance via a NS1/NS2 – PI3K/Akt pathway**

The p53 and NF-κB pathways are known to be interconnected and responsible for reciprocal functional balance at the crossroads of several cellular processes [41]. Based on previously described data on the interplay between RSV and NF-κB [25–31], we mock-infected or infected A549 cells in the conditions that lead to a marked decrease of p53 activity (m.o.i. 1, 48 h p.i., as described above), and then used a fluorescent reporter assay to evaluate the phosphorylation of p65, as a surrogate of NF-κB activity (Fig. 5a). Bacterial lipopolysaccharide (LPS) treatment was used as a positive control. In these conditions, we observed a strong and significant increase on the relative fluorescence intensity (290-fold increase, $P<0.05$) (Fig. 5a), indicating that the RSV-induced decrease of p53 activity is inversely correlated with NF-κB activity. To complete this observation, we also performed a similar experiment in the context of Nutlin-3 or DMSO (Ctrl) treatment. The stabilization and activation of p53 by Nutlin-3 in RSV-infected cells was associated with a significant reduction on fluorescence intensity compared to the 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 µg of plasmid, 48 h post transfection) inhibits p53 transcriptional activity (Fig. 5b). We observed that both NS1 and NS2 induce a strong increase of p65 phosphorylation, reflecting an up-regulation of NF-κB activity, in agreement with previously published studies [17, 32]. Altogether, these observations suggest that the NS1/NS2-induced modulation of p53...
Fig. 5. RSV infection modulates the p53/NF-κB balance via a NS2–PI3K/Akt pathway. (a) Human lung A549 cells were mock-infected or infected with RSV (Long strain) at an m.o.i. of 1 and analysed at 48 h p.i. p65 phosphorylation, considered as a reflect of NF-κB activity, was measured using a specific reporter assay and expressed as the relative value compared to the mock. Bacterial lipopolysaccharide treatment (LPS, 2 µg ml⁻¹) was used as a positive control. Measures were also performed in the context of treatment with DMSO (ctrl) or the small-molecule Mdm2 antagonist Nutlin-3 (10 µM). * , ** , *** and **** stand for P-values <0.05, 0.01, 0.001 and 0.0001, respectively. (b) A549 cells were transfected with either an empty plasmid (Empty, pEGFP-C1) or plasmids expressing RSV NS1-eGFP
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 [41]. We therefore infected A549 cells using the same viral parameters described above (m.o.i. of 1), and cell lysates were harvested at 48 h.p.i. and analysed by Western blot (Fig. 5c). In the context of RSV-infected cells, we observed an increase of the phosphorylated form of Akt, with a Akt-Pho/Total Akt relative ratio of up to fivefold in comparison with mock-infected cells (Fig. 5c). These results suggest that the PI3K/Akt could be involved in the RSV-induced down-regulation of p53 stability and consecutive 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. 3b, c) and protein level in Western blots (Fig. 3d), and suggest that NS1 and NS2 could be involved in a PI3/Akt-dependent down-regulation 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 cytokine and chemokine expression during infection. To that end, A549 cells were pre-treated 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 an antibody-based assay. 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-1RA or IL6 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).

**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 [42]. In addition, a large number of studies clearly demonstrated that this 'guardian of the genome' [43] or 'cellular gatekeeper' [44] is also involved in the control of viral infections, acting at the crossroads of several signalling pathways and cellular responses, such as inflammation and immune response (reviewed in [33, 45]). 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 [46]. 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 [37, 47–51]. However, our current understanding of the abrogation mechanisms of p53 activity in the context of RSV infection remains quite limited.

Groskreutz and colleagues have demonstrated that RSV decreases p53 levels by enhancing p53 degradation through the Akt-dependent activation of its negative regulator Mdm2 [34]. 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 [34]. 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–proteasomal-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 Groskreutz et al. [34].

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 (Figs 3 and 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, 38]. 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 [35, 36]. 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-kB in opposite ways, with a strong down-regulation of p53 transcriptional activity (Fig. 1) and a strong activation of NF-kB activity (Fig. 5a) at the same time-point during infection. The interplay between RSV and the NF-kB pathway has been extensively studied, including the contribution of NS1/NS2 proteins [17, 25–32], or more recently the small hydrophobic (SH) protein [52].

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 [41]). 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 leads certainly to a large impact on the host transcriptional programme 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. 5(f).

These new data about the interplay between RSV and p53 could be useful to complete our understanding of the role of NF-kB in the context of RSV infection. For example, it could contribute to explaining the underlying antiviral activity of some molecules with NF-κB inhibitor properties in the context of RSV infection, such as curcumin or acetylsalicylic acid [53, 54]. The functional interactions between NS proteins and the p53/NF-kB 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 favours viral replication. This regulation loop is tightly interconnected with the NF-kB 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.

**METHODS**

**Cell lines and viral strain**

Human lung epithelial A549 cells (ATCC CCL-185, wild-type p53 wt) and H1299 (ATCC CRL-5803, p53 null) were maintained at 37 °C with 5 % CO2 in Dulbecco’s modified Eagles’s medium (DMEM), supplemented with 10 % heat-inactivated fetal bovine serum, 2 mM l-glutamine, 100 U ml−1 penicillin and 100 µg ml−1 streptomycin sulphate. Human respiratory syncytial virus Long strain (ATCC VR-26) was propagated and titrated in HEp-2 cells (ATCC CCL-23). For infection, m.o.i. were based on infectious titres (TCID50 ml−1).

**Stability, transactivation and RT-qPCR assays**

For the determination of p53 half-life, cells were treated with cycloheximide (50 µg ml−1), and total protein lysates were harvested at different time points and then analysed by Western blot. p53 relative protein levels (RPL) were determined by densitometry analysis using the ImageJ software (https://imagej.nih.gov/ij/), as previously described [47]. For transactivation assays, cells were transfected with 1 µg of pG13-luc vector, harbouring the firefly luciferase gene under the control of 13 copies of the p53-binding consensus sequence (5′-CCAGGCAAGCTCCAGGAG-3′) [55]. 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 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 p53 mRNA levels was performed by RT-qPCR as previously described [47]. 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, CA, USA) according to the manufacturer’s instructions. Samples with a cycle threshold (Ct) value of ≥40 were recorded as negative. Genome copies ml⁻¹ 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 1 x NuPAGE LDS buffer (Invitrogen). In total, 15–30 µg 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 (no. 9792, Santa Cruz Biotechnology), rabbit polyclonal anti-Phospho Akt (ser473) (no. 4058, Santa Cruz Biotechnology). In addition, an anti-Ku80 polyclonal antibody was used as a loading control (no. 2753, Cell Signaling).

**Plasmid and siRNA transfection**

Plasmid transfections were performed using TransIT-LT1 reagent (Mirus), according to the manufacturer’s instructions. Transfection efficiencies were estimated of 10–15% after 24 h and 60–70% after 48 h in Lab-Tek II chamber slides (ThermoScientific, Fluorescence immunostaining experiments) and in multi-well six plates (Western blot and transactivation experiments), respectively. N51-EGFP and N52-EGFP expression plasmids, (pEGFP-C1 plasmid) [39] were graciously obtained from Ralph Tripp (Department of Infectious Diseases, University of Georgia). pSV-p53 expression plasmid was a kind gift from Dr Jean-Christophe Bourdon (Division of Cancer Research, University of Dundee, UK). As indicated in figure legends, 0.2 to 2 µg of plasmid was transfected in 0.3.10⁶ cells A549 or H1299 cells. Silencing of p53 was performed in A549 cells transfected with a siRNA specifically targeting p53 (si-p53) [48] and a non-specific siRNA (si-Control, OR-0030-neg05, Eurogentec), as a control, by using Oligofectamine (Invitrogen), according to the manufacturer’s instructions. In the context of infections (mock or RSV-infected cells), plasmid and siRNA transfections were performed 48 h before infection.

**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 1 h incubation, cells were washed in PBS-T and then incubated with goat anti-mouse coupled to AlexaFluor 633 (Molecular Probes, Invitrogen), as a control, by using Oligofectamine (Invitrogen), according to the manufacturer’s instructions. In the context of infections (mock or RSV-infected cells), plasmid and siRNA transfections were performed 48 h before infection.

**NF-κB and cytokine/chemokine immunoassays**

Cell culture supernatants were analysed for the presence of 27 human cytokines and chemokines, as previously described [56], using the Bio-Plex Pro Human Cytokine Standard 27-Plex kit (Bio-Rad) on a FLEXMAP 3D analyser (Luminex, Austin, Texas, USA). The same technology was used for the monitoring of p65 phosphorylation, using the Bio-Plex Pro Phospho-NF-κB p65 kit (ser536) (BioRad).

**Reagents**

Small molecule Mdm2 antagonist Nutlin-3a (Calbiochem) was dissolved in DMSO, aliquoted and stored at −20 °C. A549 cells were infected by RSV at an m.o.i. of 1, in the presence of DMSO (Ctrl) or a small-molecule Mdm2 antagonist (Nutlin-3, 10 µM). Protein synthesis inhibitor cycloheximide (50 µg ml⁻¹) and proteasome inhibitor MG132 (20 µM) were purchased from Sigma-Aldrich (refs C7698 and M8699, respectively).

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**

8. Zwaans WA, Mallia P, van Winden ME, Rohde GG. The relevance of respiratory viral infections in the exacerbations of chronic


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