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Activation of p21 limits acute lung injury and induces early senescence after acid aspiration and mechanical ventilation

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The p53/p21 pathway is activated in response to cell stress. However, its role in acute lung injury has not been elucidated. Acute lung injury is associated with disruption of the alveolo-capillary barrier leading to acute respiratory distress syndrome (ARDS). Mechanical ventilation may be necessary to support gas exchange in patients with ARDS, however, high positive airway pressures can cause regional overdistension of alveolar units and aggravate lung injury. Here, we report that acute lung injury and alveolar overstretching activate the p53/p21 pathway to maintain homeostasis and avoid massive cell apoptosis. A systematic pooling of transcriptomic data from animal models of lung injury demonstrates the enrichment of specific p53- and p21-dependent gene signatures and a validated senescence profile. In a clinically relevant, murine model of acid aspiration and mechanical ventilation, we observed changes in the nuclear envelope and the underlying chromatin, DNA damage and activation of the Tp53/p21 pathway. Absence of Cdkn1a decreased the senescent response, but worsened lung injury due to increased cell apoptosis. Conversely, treatment with lopinavir and/or ritonavir led to Cdkn1a overexpression and ameliorated cell apoptosis and lung injury. The activation of these mechanisms was associated with early markers of senescence, including expression of senescence-related genes and increases in senescence-associated heterochromatin foci in alveolar cells. Autopsy samples from lungs of patients with ARDS revealed increased senescence-associated heterochromatin foci. Collectively, these results suggest that acute lung injury activates p53/p21 as an antiapoptotic mechanism to ameliorate damage, but with the side effect of induction of senescence. (Translational Research 2021; 233:104–116)

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Submitted for Publication August 14, 2020; received submitted December 13, 2020; accepted for publication January 21, 2021.

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1931-5244/$ - see front matter

https://doi.org/10.1016/j.trsl.2021.01.008

Abbreviations: ANOVA = analysis of the variance; ARDS = acute respiratory distress syndrome; SAHF = senescence associated heterochromatin foci; SASP = senescence-associated secretory phenotype; TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling; VILI = ventilator-induced lung injury

AT A GLANCE COMMENTARY

Jorge Blázquez-Prieto et al.

Background

P53 and its downstream factor p21 are involved in the cell response to stress. In addition, they are drivers of senescence. Although these pathways have been studied in chronic lung diseases, their role in acute lung injury has not been systematically explored.

Translational Significance

Our findings show that the antiapoptotic effects of p21 counteract the proapoptotic response triggered by acute lung injury and mechanical ventilation. Drug-induced overexpression of p21 decreases lung injury in this setting. However, activation of p21 also leads to an early senescent response within the lung that may favor long-term side effects.

INTRODUCTION

The lungs have a stereotypic response to acute injury, which is preserved among species and many etiological agents. Once damage is inflicted, lung cells trigger a host response which can include inflammation, matrix remodeling and different forms of cell death, including apoptosis.[1] Although a limited host response may help to clear the injurious agent and promote lung tissue repair,[2] an overexuberant host response can lead to severe injury and gas exchange worsening. Therefore, therapeutic strategies aimed to limit lung damage and interference with lung repair are important.

Lungs are exposed to mechanical load during every breath. In pathologic conditions, generation of higher-pressure gradients necessary for adequate ventilation may cause excessive cell stretch.[3] This is especially relevant during mechanical ventilation with high pressures, which can lead to the so-called ventilator-induced lung injury (VILI).[4,5] In mechanically ventilated patients, a strategy aimed to limit VILI decreased mortality in patients with the acute respiratory distress syndrome (ARDS).[6]

Mechanotransduction is thought to regulate the molecular steps in VILI pathogenesis.[7] The nuclear envelope has been reported as an important cell mechanosensor and signal transducer.[8] Mechanical stretch appears to increase Lamin-A in the nuclear envelope, leading to nuclear stiffening. These changes in the nuclear envelope can also activate p53-dependent pathways. Wildtype p53 is a master regulator of cell homeostasis and fate, and its activation may lead to a variety of responses, ranging from apoptosis to cell cycle arrest. Inhibition of this response has been shown to increase p21 (Cdkn1a) expression and decrease VILI in an experimental model.[9] p53 and its downstream factor p21 are triggers of senescence,[10] a cell response characterized by an stable arrest of the cell cycle and a switch towards a senescence-associated secretory phenotype (SASP). It has been proposed that senescence facilitates the clearance of damaged cells and is required for tissue repair.[11] Interestingly, some of the molecules have a significant overlap with the proinflammatory response associated with VILI.

We hypothesized that p53-dependent pathways play a role in the maintenance of lung homeostasis during acute injury, and that senescence could be a side effect of their activation. To test this hypothesis, we developed a clinically relevant model of lung injury caused by acid aspiration and VILI to assess the activation of p53 and its downstream factors.

MATERIAL AND METHODS

Meta-analysis of transcriptomic data. To explore the main hypothesis, a pooled analysis of published transcriptomic data was performed, using a previously validated 55-gene expression signature of senescence[12] as main endpoint. Datasets reporting lung gene expression in mouse models of acute lung injury and mechanical ventilation were obtained from public repositories (Gene Omnibus Expression -https://www.ncbi.nlm.nih.gov/geo/- and ArrayExpress - https://www.ebi.ac.uk/arrayexpress/-) using the following terms: "Stretch," "Cyclic strain," "Mechanical Ventilation," "Lung," and "Alveolar." Fifty-one datasets were manually reviewed. Studies lacking a control group with intact, spontaneously breathing animals and those reporting less than 40 genes from the endpoint signature were excluded, so 9 datasets were finally used (Supplementary Table 1). When available, raw data was downloaded and normalized using the Robust Multiarray

Average method (for Affymetrix microarrays) or normal-exponential background correction followed by quantile normalization (all the other platforms).

Normalized datasets were pooled using the Combat-Co-normalization using controls (COCONUT) algorithm.[13] This method normalizes gene expression of the different datasets using an empirical Bayes fitting, but applied only to control samples (in this case, spontaneously breathing animals with intact lungs). Then the obtained normalization parameters are applied to the cases (ie, those with lung injury) (Supplementary Fig 1). Three different signatures were studied, corresponding to 116 genes upregulated by p53, 14 genes downregulated by p21[14] and a set of 50 genes consistently up- and down-regulated in senescence.[12] A meta-score was computed for each sample as the geometric mean of the upregulated genes minus the geometric mean of the downregulated genes in the signature.[13] Meta-scores were finally compared among controls and animals with lung injury and/or mechanical ventilation.

Animal models. Male, 12-week-old C57Bl/6 mice, kept under pathogen-free conditions with free access to food and water, were used in all experiments. The Animal Research Committee of the Universidad de Oviedo evaluated and approved the study.

A 2-hit lung injury model, based on chlorhydric acid instillation and mechanical ventilation, was studied. Animals were anesthetized with intraperitoneal ketamine and xylazine and orotracheally intubated using a 20G catheter, through which 50 μL of chlorhydric acid (0.1N, pH = 1.5) were instilled. Two hours after instillation, mice were randomly assigned to receive mechanical ventilation or not. Mice were ventilated with a pressure-controlled mode (peak inspiratory pressure 17 cm H_2O, PEEP 2 cm H_2O, respiratory rate 100 breaths/min) for 120 minutes.

Three additional series of experiments were performed. Mice lacking Tp53 or Cdkn1a (p21, an endogenous inhibitor of cyclin-dependent kinases involved in the senescent response triggered by Tp53) and their wildtype littermates were subjected to the same model of injury, including acid instillation and mechanical ventilation. Genotypes were confirmed by PCR. In separate experiments, wildtype animals were treated with a single dose (200/50 mg/Kg) of lopinavir/ritonavir (a protease inhibitor that inhibits Zmpste24 and disrupts Lamin-A nuclear scaffolding, activating senescence pathways) or saline, administered intraperitoneally immediately after acid instillation, and then ventilated with the parameters described above.

Tissue harvest. Mice were studied in 3 different conditions: baseline, 4 hours after chlorhydric acid instillation without mechanical ventilation and 4 hours after acid instillation including 2 hours of mechanical ventilation. Lungs were removed after exsanguination of anesthetized animals. A laparotomy was performed, the renal artery sectioned, the thorax opened and the heart-lungs removed in bloc. The left lung was instilled with 250 microliters of 4% phosphate-buffered paraformaldehyde, immersed in the same fixative for 24 hours, and then stored in 50% ethanol. The right lung was immediately frozen at −80˚C for biochemical analyses.

Patient samples. Paraffin-embedded lung tissue from autopsies of patients were obtained from the tissue bank at Hospital Universitario Central de Asturias, after signed consent from patients' next of kin. ARDS was defined using the Kigali modification of the Berlin definition,[15] to include patients with lung injury but without mechanical ventilation and those without an arterial line. Thirteen samples were recovered (Supplementary Table 2).

Histological studies. After fixation, tissues were embedded in paraffin and 3 slices with at least 1mm of separation between them were cut and stained with hematoxylin and eosin. A pathologist blinded to the experimental settings evaluated the degree and extension of lung damage using a predefined histological score.[16]

Additional lung sections were processed as previously described[17] for detection of myeloperoxidase- and Ki-67-positive cells, using specific antibodies (See Supplementary Table 3 for references). Images from 3 random fields (x200) were taken and then number of positive cells averaged.

For immunofluorescence studies, slides were deparaffinated and antigens retrieved in citrate buffer 0.1M (pH = 9). The autofluorescence of the tissue was diminished using a Sudan black B solution and sections were permeabilized (0.1% Triton X-100 in PBS for 15 minutes), blocked (1% BSA in PBS) and incubated overnight at 4˚C with the primary antibody (Supplementary Table 3). After 24 hours, the slices were incubated with the corresponding secondary fluorescent antibody at room temperature for 1 hour. Images were taken using a confocal microscopy (Leica SP8) at 400x and 630x. The number of positive and negative nuclei were automatically quantified using ImageJ software (NIH, Bethesda, Maryland, USA).

Apoptotic cells in lung slices were detected by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) as previously described.[9] Images from 3 random fields were acquired in a Leica SP8 confocal microscope and the positive nuclei were counted and expressed as percentage of the total nuclei count.

Western blot. Nuclei were extracted from fresh lung tissues and subsequently homogenized as described

before.[9] The total amount of protein from nuclear extracts was quantified (BCA Protein Assay Kit, Pierce) and 15μg of each sample was loaded in SDS-polyacrylamide gels, electrophoresed at 120mV and electrotransferred onto PVDF membranes. After blockade with 5% non-fat dry milk, the membranes were incubated with primary antibodies against Caspase-9, Lamin-A/C, Lamin-B1, γH2AX, HP1α or H3 (Supplementary Table 3) in 3% nonfat dry milk overnight at 4°C. After 24 hours, the membranes were incubated with the corresponding peroxidase-conjugated secondary antibodies in 2.5% non-fat dry milk. Proteins were detected by chemiluminescence in a LAS-4000 Imaging system. The intensity of each protein band was quantified using ImageJ software (NIH).

Quantitative PCR. Lung fragments (2 mm x 2 mm) were homogenized with TRIZOL (Sigma, Poole, UK) and RNA precipitated by overnight incubation in isopropanol at −20°C. After 24 hours, samples were washed with ethanol and the RNA resuspended in RNAse-free water and quantified. One μg of total RNA was retrotranscribed into complementary cDNA using an RT-PCR kit (High-capacity cDNA rt Kit, Applied Biosystems). Quantitative PCRs were carried out in triplicate of each sample using 40 ng of cDNA per well. Expression of Plk3, Gdnf, Meis1, Il6, Tp53, Cdkn1a (p21), Cdkn2a (p16), Rb, and Gapdh was quantified using Sybr-green Power up, (Fisher Scientific) and 10uM of the corresponding primers (Supplementary Table 4). The relative expression of each gene was calculated as $2^{-\Delta CT(\text{gene of interest})-\Delta CT(GAPDH)}$.

Statistical analysis. Data are shown as mean ± standard error of the mean. Differences between 2 groups were studied using a T test. Differences among more than 2 groups were assessed using an analysis of the variance (ANOVA). For SAHF counts, 3 slides per animal were counted (considered as technical replicates) and analyzed using a mixed-effects ANOVA. When significant, pairwise comparisons were done using the Tukey's Honest Significant Difference test. A P value lower than 0.05 was considered significant.

RESULTS

Transcriptomic signatures of p53/p21 activation and senescence in lung injury. To test the hypothesis that the p53/p21 pathway is activated during acute lung injury and to identify early markers of a switch towards a senescent phenotype, data from 9 datasets of mouse lung injury and mechanical ventilation (Supplementary Table 1) were pooled and gene expression analyzed (Fig 1, A). Three different transcriptomic signatures related to p53-dependent upregulation (116 genes, 85 available in the pooled data), p21-dependent downregulation (14 genes, 12 available)[14] and senescence[12] (55 genes, 44 available) were analyzed. A meta-score of expression of these genes was computed for each sample and compared to assess the effect of lung injury and mechanical ventilation.

Animals subjected to acid aspiration lung injury and mechanical ventilation showed higher expression of p53-dependent genes (ANOVA p-value<0.001, Fig 1, B), lower expression of p21-downregulated genes (ANOVA P value <0.001, Fig 1, C) and a higher meta-score (Fig 1, D) in the senescence signature than spontaneously breathing controls (ANOVA P value <0.001, Fig 1, D). The expression of each gene of these signatures is shown in Supplementary Fig 2. These results support the notion that lung injury and mechanical ventilation activate p53/p21 pathways and the molecular mechanisms of senescence in acutely injured lungs.

Activation of the p53/p21 pathway in a clinically relevant model. To explore the mechanisms involved in the activation of p53-dependent signals, an experimental model of acid aspiration- and mechanical ventilation induced lung injury was tested. Chlorhydric acid instillation and mechanical ventilation induced a significant increase in lung damage and inflammation, assessed by histological scores (Fig 2, A), neutrophilic infiltrates (Fig 2, B) and Il6 expression (Fig 2, C). Lung damage increased the proportion of both proliferating and apoptotic cells in lung parenchyma (Fig 2, D and E and Supplementary Fig 3). Immunohistochemical studies revealed that TUNEL-positive staining was localized in all the explored cell types, including type I and II pneumocytes, fibroblasts and endothelial cells (Supplementary Fig 4). In line with these findings, the abundance of cleaved caspase-9 in lung tissue was increased with lung injury (Fig 2, F)

We then explored the putative activators of this response to acute injury. Lamins in the nuclear envelope act as cell mechanosensors, regulating chromatin organization in response to mechanical stress. We observed that Lamin-A/Lamin-B ratio increased after mechanical stretch (Fig 2, G). Immunofluorescence studies confirmed the increase in Lamin-A in the nuclear envelope after mechanical stretch, but not after hydrochloric acid instillation alone (Fig 2, H). These changes in the nuclear envelope coexisted with an increase in γH2AX (Fig 2, I) and HP1α (Fig 2, J), markers of DNA damage and chromatin remodeling respectively, in nuclear extracts from ventilated animals. Panel 2K shows representative Western blots of these parameters.

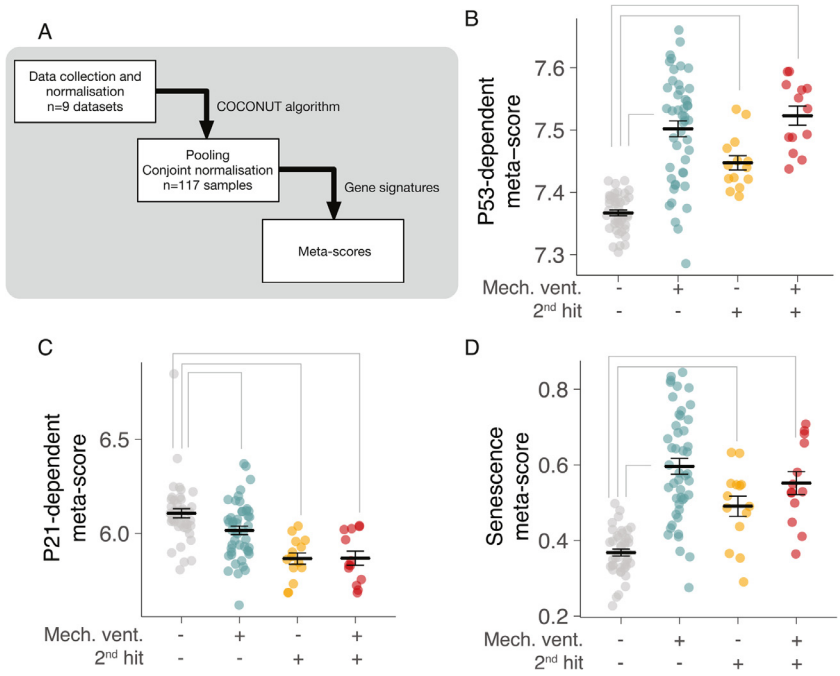

Fig 1. Expression of gene signatures. **A**, Overview of the analysis. Eleven datasets (128 samples) reporting gene expression in animal models of lung injury were pooled and analyzed to calculate different Meta-scores summarizing the expression of genes included in specific signatures. **B**, Meta-score of a p53-dependent signature for each experimental group (second hit refers to any model of lung injury other than mechanical ventilation). **C**, Meta-score of a transcriptomic signature including genes downregulated by p21. **D**, Meta-score of a senescence-specific signature. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc tests).

The expression of the canonical responders to DNA damage Cdkn2a (p16) and Tp53 (p53) and their corresponding downstream factors Rb and Cdkn1a (p21) was also assessed. There were no differences in the levels of Cdkn2a (Fig 2, L), whereas expression of Rb was significantly decreased (Fig 2, M). However, we observed significant increases in Tp53 (Fig 2, N) and Cdkn1a (Fig 2, O) expression with lung injury. The increase in P21 protein was observed mainly in type-I and type-II alveolar epithelial cells (positive for Aquaporin-5 or Surfactant-protein C respectively), and not in fibroblast or endothelial cells (positive for vimentin or Von-Willebrand factor respectively) (Supplementary Fig 5).

Increased lung damage in mice lacking p21. To address the role of p53 and p21 in acute lung damage, $Tp53^{-/-}$, $Cdkn1a^{-/-}$ mice and their wildtype counterparts were subjected to acid instillation followed by mechanical ventilation. In preliminary experiments, absence of Tp53 did not modify lung injury (histological score 2.4 ± 1.6 vs 2.3 ± 1.2, n = 4/group, $P = 0.91$, Supplementary Fig 6), so we focused on the downstream factor p21. In absence of p21, the mice had worse lung injury (Fig 3, A) and higher counts of apoptotic cells (Fig 3, B) and cleaved caspase-9 abundance (Fig 3, C) compared to their wildtype counterparts. There were no differences in Il6 or Tp53 expression nor in abundance of γH2AX or HP1α between genotypes (Fig 3, D–G respectively).

Lopinavir increases p21 and decreases lung damage. We have previously shown that HIV-protease inhibitors modify the nuclear response to mechanical stretch and protect against VILI,[9] an effect that could be due to the inhibition of the Lamin-A protease ZMPSTE24.[18] In our double-hit model, treatment with lopinavir and/or ritonavir impaired the structure of the nuclear lamina, decreasing the abundance of Lamin-A (Fig 4, A), and decreased lung injury (Fig 4, B), apoptotic cell count (Fig 4, C) and cleaved caspase-9 (Fig 4, D). Although abundance of γH2Ax was not modified by this treatment (Fig 4, E), there was a marked decrease in HP1α (Fig 4, F). Panel 4G shows representative blots of these measurements. Finally, treatment with lopinavir and/or ritonavir caused an increase in Il6 expression (Fig 4, H), with no changes in Tp53 expression (Fig 4, I) but an increase in Cdkn1a (p21, Fig 4, J).

Early markers of senescence in acute lung injury. Then, we tried to identify early markers of senescence in our acute model. Acid aspiration and mechanical ventilation-induced lung injury was associated with an increase in the number of nuclei positive for Macro-

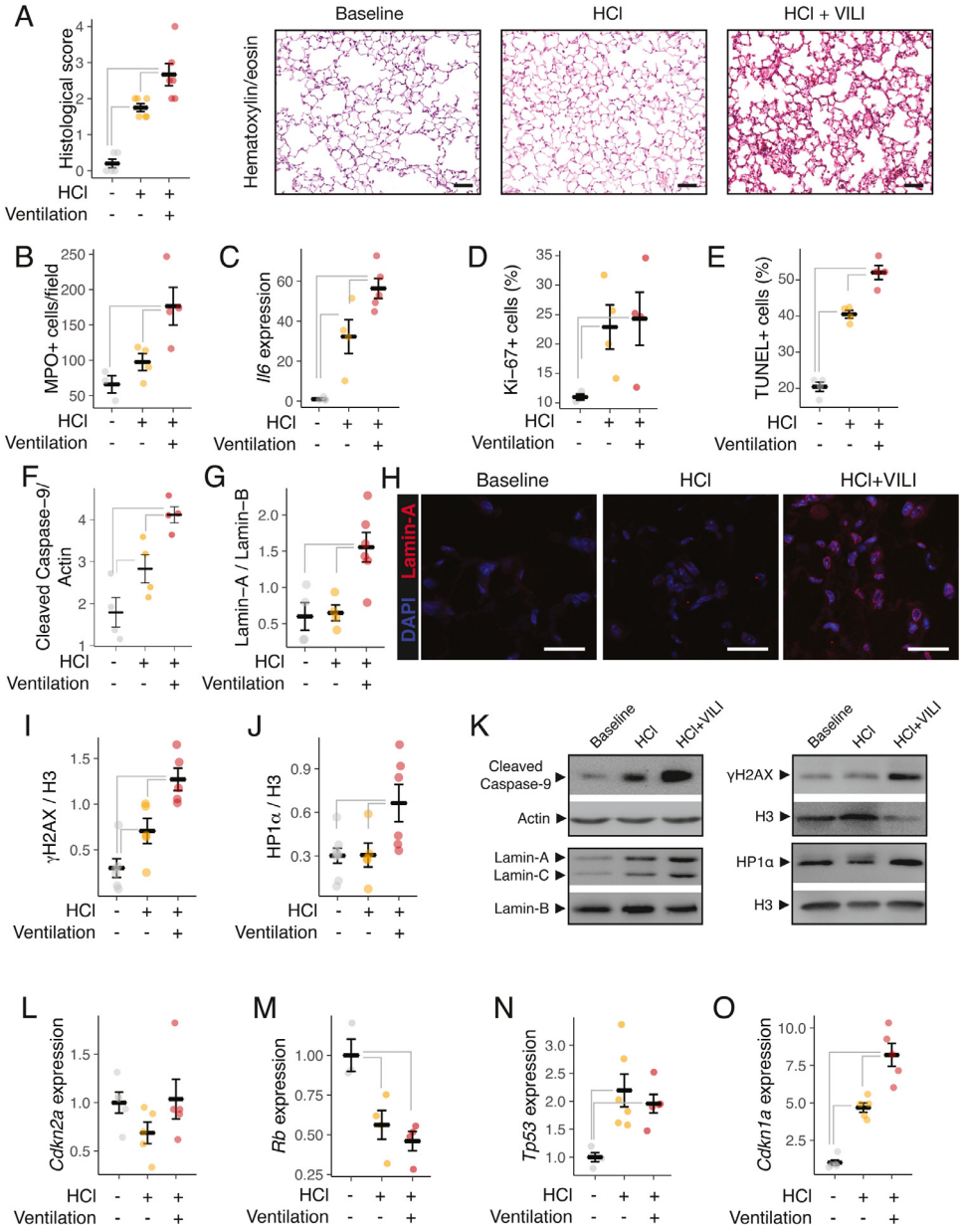

Fig 2. Characterization of lung injury. **A**, Acid instillation and mechanical ventilation caused lung damage assessed using a histological score (scale bar:100 μ). **B**, Myeloperoxidase-positive cell counts in histological sections, showing an increase of neutrophils in the injured lung. **C**, Expression of Il6 in lung tissue. **D**, Quantification of Ki-67 positive cells in histological sections, as a marker of proliferation. **E**, TUNEL-positive cells in histological sections. **F**, Abundance of cleaved Caspase-9 in lung homogenates. **G**–**H**, Changes in Lamin-A/Lamin-B1 ratio in nuclei from lung tissue (**G**) and representative immunohistochemical sections (**H**, scale bar: 25 μ). (**I**–**J**) Abundance of γH2AX (**I**) and HP1α (**J**), markers of DNA damage and heterochromatin respectively, in nuclei from lung tissue. **K**, Representative western blots of the previous quantifications. **L**–**O**, Changes in expression of the canonical senescence inducers Cdkn2a (p16, **L**), Rb (**M**), Tp53 (**N**) and Cdkn1a (p21, **O**). N = 4–6 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc tests).

H2A, a marker of senescence-associated heterochromatin foci (SAHF, Fig 5, A), and changes in Plk3 (Fig 5, B), Gdnf (Fig 5, C), and Meis1 (Fig 5, D), the genes from the senescence signature with the highest differential expression in the previous pooled analysis.

These markers of senescence were modified by manipulation of the p21 pathway. Mutant animals lacking Cdkn1a exhibited a decreased number of SAHF after acid instillation and mechanical ventilation (Fig 5, E) and in expression of the senescence-related

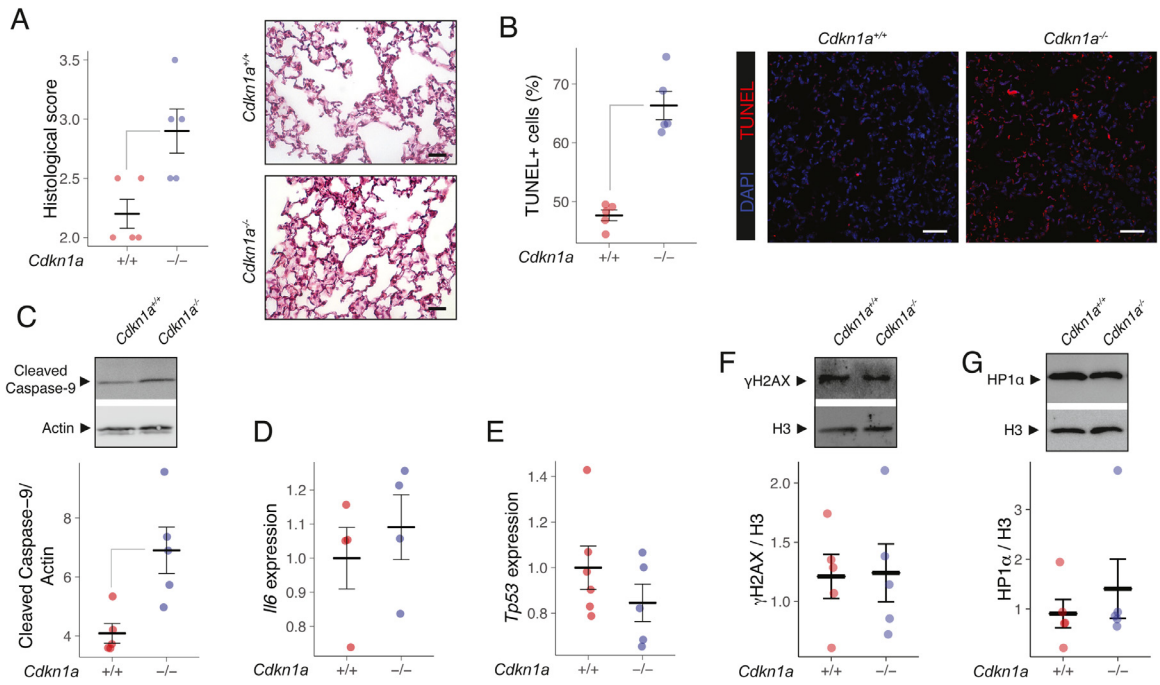

Fig 3. Lung injury in wildtype and Cdkn1a$^{-/-}$ animals. **A**, Histological score of lung damage in both genotypes (scale bar: 100 μ). **B**, Percentage of apoptotic (TUNEL+) cells (scale bar: 50 μ). **C**, Abundance of cleaved caspase-9 in lung homogenates from both genotypes. **D**−**E**, Expression of Il6 (**D**) and Tp53 (**E**) in wildtype and mutant mice. **F**−**G**, Abundance of γH2AX (**F**) and HP1α (**G**), with representative western blots, in lung homogenates. N = 4−6 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in T tests).

gene Plk3 (Fig 5, F). In opposite, treatment with lopinavir and/or ritonavir (that increased Cdkn1a expression, Fig 4, J) was related to lower counts of SAHF (Fig 5, G), but increased Plk3 expression (Fig 5, H).

Finally, to confirm the incidence of SAHF in patients, lung tissue from autopsies of critically-ill patients with and without lung injury and mechanical ventilation (Supplementary Table 2) were stained with antibodies against Macro-H2A. There were no differences in age between the 3 groups of patients (62 ± 6, 61 ± 11, and 54 ± 10 years for patients without ARDS or mechanical ventilation, with ARDS but without mechanical ventilation and ARDS and ventilation respectively, $P = 0.17$ in ANOVA). Similarly, to the animal model, nuclear Macro-H2A increased in those with severe lung injury and mechanical ventilation (Fig 5, I).

Collectively, these findings suggest that lung injury and mechanical stretch trigger the appearance of early markers of senescence. The severity of lung injury and the abundance of senescence markers showed an inverse correlation after manipulation of p21 levels.

DISCUSSION

We provide evidence that acute lung injury and its treatment with mechanical ventilation alters the nuclear envelope and causes DNA damage, activating the p53/p21 pathway. Activation of p21 plays a homeostatic role, limiting the extent of apoptosis in response to injury. Moreover, this effect can be pharmacologically activated to ameliorate lung injury in a clinical setting. In spite of this beneficial effect, this pathway also leads to the appearance of early markers of senescence in lung tissue. Fig 6 summarizes the findings of this work.

The p53/p21 axis in acute injury. P53 and its downstream transcription factor p21 are major regulators of cell homeostasis. It has been shown that p53 regulates permeability in lung endothelial cells after an inflammatory insult.[19] Similarly, activation of this pathway in response to hypertonic saline decreased lung injury and inflammation in human airway epithelial cells.[20] However, our observations in Tp53$^{-/-}$ mice showed no differences in lung injury. Given the pleiotropic effects of p53 in cell homeostasis, this could be due to the existence of both protective and pathogenetic mechanisms.

One of the main effects of the cyclin kinase inhibitor p21 is the blockade of apoptosis.[21] Several

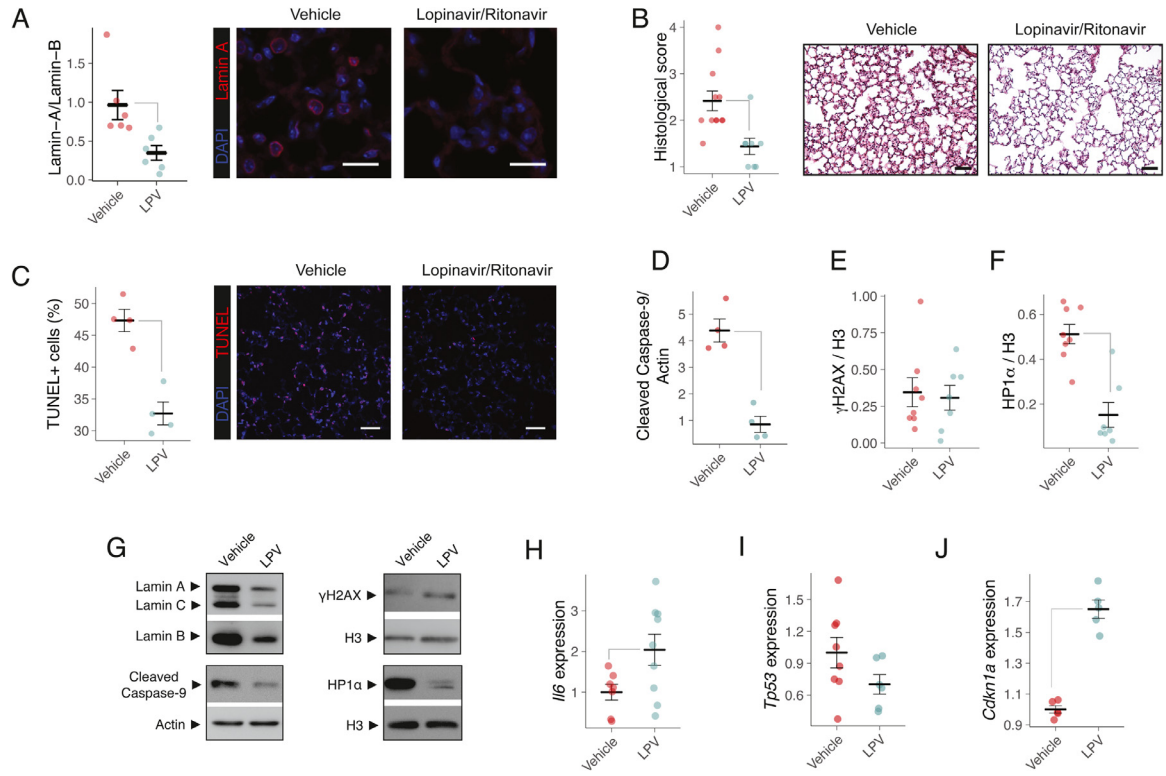

Fig 4. Effects of Lopinavir/Ritonavir on lung injury. **A**, Lamin-A abundance and staining in vehicle- and lopinavir/ritonavir treated animals (scale bar: 25 μ). **B**, Histological score of lung damage (scale bar: 100 μ). **C**, Apoptotic (TUNEL+) cell counts in both groups (scale bar: 50μ). **D-F**, Abundance of Caspase-9 in tissue homogenates (**D**), γH2AX (**E**) and HP1α (**F**), with representative western blots (**G**) in lung homogenates. **H**–**J**, Expression of Il6 (**H**), Tp53 (**I**) and Cdkn1a (p21, **J**). N = 7–10 animals per group. Gray lines mark significant differences among groups ($P < 0.05$ in T tests).

proapoptotic pathways are activated during acute lung injury,[22] as shown in our model. In this setting, p21 may have a compensatory role by avoiding the loss of a large amount of epithelial cells. Our observation of massive cell death in Cdkn1a$^{-/-}$ mice suggests this homeostatic role. It has been proposed that caspase-9 is the downstream target responsible for the antiapoptotic effects of p21.[23] Overexpression of p21 increases the resistance to apoptosis of alveolar epithelial cells,[24] and the beneficial effects of lopinavir and/or ritonavir in VILI could represent the effects of the overexpression of this gene and the observed decrease in caspase-9. In contrast, absence of p21 was associated with more severe lung injury and increased numbers of apoptotic cells, as previously suggested.[25]

The role of mechanical stretch. Mechanical overstretch could be an important pathogenic factor involved in p53/p21 activation. The experimental model using a ventilatory strategy within the limits of protective ventilation (driving pressure 15 cm H_2O, avoidance of zero end-expiratory pressure) was chosen to increase the translational significance of our work. Although ventilation with very high inspiratory pressures and allowing expiratory collapse induces severe lung damage in less than 1 hour, even in healthy lungs, the clinical translation of these findings is less straightforward.

Several mechanisms have linked the mechanical load to a lung biological response, including oxidative stress and MAPK activation.[26] We focused on the role of the nuclear envelope as a critical structure regulating both mechanosensing and senescence. The mechanical load is transmitted from the extracellular matrix to the cytoskeleton and then to the nuclear membrane.[27] This causes a change in the nuclear lamina, reorganization of the underlying chromatin and DNA damage,[28] either mediated by MAPK activation[29] or by a direct mechanical effect.[30] DNA damage is one of the triggers of the p53 pathway. In smooth muscle cells, stretch leads to p53 activation and upregulation of senescence markers,[31] resembling our findings. Similarly, HIV protease inhibitors, such as lopinavir and/or ritonavir, inhibit ZMPSTE-24, a protease responsible for Lamin-A maturation,[18] preserving nuclear compliance, increasing p21 expression and decreasing stretch induced apoptosis and VILI.[9]

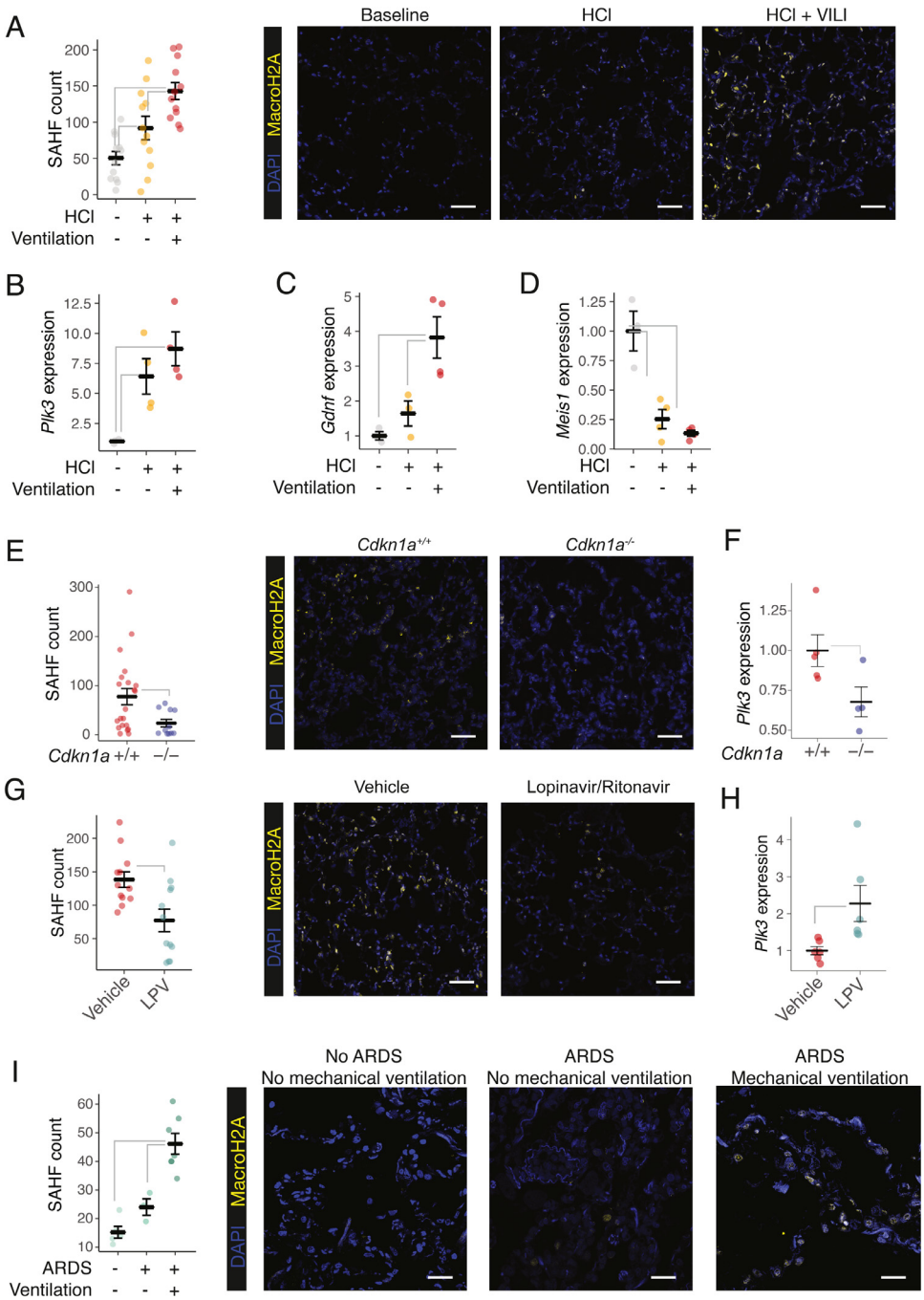

Fig 5. Identification of early senescence markers in experimental models and patients. **A**, Counts of Senescence-associated heterochromatin foci (SAHF) in the experimental model of lung injury of acid instillation and mechanical ventilation (scale bar: 50μ). **B**–**D**, Expression of Plk3, Gdnf, and Meis1 in lung tissue. These senescence-associated genes were identified in the genomic analysis as those with the largest differences between control and injured samples. **E**–**F**, SAHF counts (**E**) and Plk3 expression (**F**) in wildtype and Cdkn1a$^{-/-}$ mice after lung injury. **G**–**H**, SAHF counts (**G**, scale bar: 25μ) and Plk3 expression (**H**) in vehicle and lopinavir/ritonavir (LPV)-treated mice after lung. **I**, Appearance of SAHF in autopsy samples from critically ill patients who died in the Intensive Care Unit with or without mechanical ventilation and acute respiratory distress syndrome (ARDS) (scale bar: 50μ). N = 4–7 animals per group, with 3 slides per animal as technical replicates in SAHF counts. Gray lines mark significant differences among groups ($P < 0.05$ in Tukey's post hoc or in T tests).

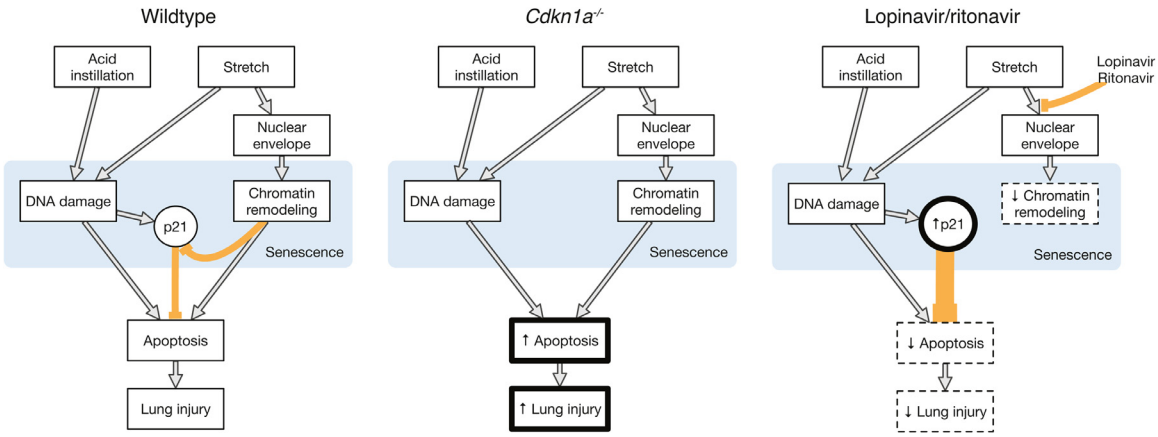

Fig 6. The role of p21 pathway on apoptosis and senescence after acute lung injury. **A**, In control mice, lung injury and mechanical stretch cause DNA damage and changes in the nuclear envelope, activating the cell senescence program. The amount of apoptotic cells depends on the equilibrium between the activation of proapoptotic responses triggered by injury itself and the antiapoptotic effects of the senescence inducer Cdkn1a (p21). **B**, In mice lacking Cdkn1a, absence of this antiapoptotic factor leads to an increase in apotosis and a more severe lung injury. **C**, Treatment with Lopinavir/ritonavir blocks the Lamin-A mediated chromatin remodeling, triggering a senescence-like response that increases p21 expression, thus decreasing apoptosis and lung damage.

Our immunohistochemical studies suggest that although apoptosis occurs in all the explored cell lines (alveolar epithelium, endothelium and fibroblasts), activation of p21 pathway take place in the alveolar epithelium. Preexisting epithelial damage caused by acid instillation could amplify the mechanical load over the epithelium by increasing heterogeneity of the lung parenchyma. Moreover, previous results in subacute models of lung damage have shown the need for 2 synergic hits to induce lung senescence.[32]

Senescence in lung diseases. One of the known consequences of p53 activation is the cell switch towards a senescent phenotype. Lipopolysaccharide or bleomycin-induced lung injury increases the number of SA-β-galactosidase-positive cells and leads to cell cycle arrest.[33] It has been shown that this activation has no detrimental effects in acute inflammation. However, blockade of the cell cycle has been associated with increased collagen deposition[34] and SASP may perpetuate lung inflammation.[35] Therefore, main features of abnormal lung repair after acute injury (limited cell proliferation, chronic inflammation and fibrosis) could be explained by a persistent senescent response.[36] Inhibition of this response by selective deletion of Tp53 in Club cells ameliorated lung damage related to chronic inflammation,[33] suggesting a novel mechanism amenable to treatment of lung diseases.

The acute nature of our model and its short-term lethality does not allow the identification of canonical senescence markers such as Senescence-associated β-galactosidase, as these require from days to weeks to be positive.[37] However, we identified a set of early markers including changes in chromatin structure and gene expression. In a model of repair after VILI, lung Cdkn1a expression remained elevated up to 2 days of spontaneous breathing after injury.[9] Although it is unclear if these mechanisms may precipitate a full-blown senescent response in the long term, our results highlight the involvement of this molecular machinery in the early phase, and could be a therapeutic target to avoid late consequences.

Clinical implications. Our findings have several implications regarding the pathogenesis of lung injury and its long-term consequences. First, the described p21 response may be beneficial in the acute phase, and could be pharmacologically manipulated using lopinavir. In a recent clinical trial in patients with lung disease caused by the SARS-CoV-2 coronavirus, lopinavir did not reduce mortality, but decreased the risk of ARDS development.[38] However, the associated senescent response could worsen lung repair and long-term outcomes. Survivors after a prolonged ICU stay may have deleterious and prolonged sequels, including respiratory impairment,[39] neuropsychological disturbances[40] and muscle atrophy,[41] particularly the elderly.[42] The mechanisms responsible for these sequels are largely unknown and no effective therapies are currently available. Local activation of senescence and its paracrine and/or systemic spread could contribute to the pathogenesis of these sequels.[43] As previously discussed, senescence may contribute to disordered lung repair. The confirmation of this framework could lead to the use of senolytics[44(p)] in critically ill patients. However, due to the protective nature of

senescence in the early phase,[45] these treatments should be time-coordinated and modulated to optimize their effectiveness.

CONCLUSIONS

We provide new evidence suggesting that acute lung damage activates p21 to limit apoptosis. This response appears to be trigged by the induction of DNA damage and linked to chromatin changes caused by mechanical overstretch. Interaction with the nuclear lamina may enhance this antiapoptotic response. Although p21 activation may be beneficial in the acute phase of lung injury, the long-term effects must be taken into consideration as they could explain some of the long-term sequels of critically ill patients.

DATA STATEMENT

Raw data and R code used in this work are available from the corresponding author (GMA) upon reasonable request.

ACKNOWLEDGMENTS

Conflicts of Interest: All authors have read the journal's policy on disclosure of potential conflicts of interest and have none to declare.

Funding: Supported by grants from Centro de Investigación Biomédica en Red (CIBER) and Instituto de Salud Carlos III (PI20/01360, Co-funded by European Regional Development Fund/European Social Fund). L.A.R. is the recipient of a grant from Instituto de Salud Carlos III (CM16/00128). C.H.F. is the recipient of a grant from Instituto de Salud Carlos III (CD16/00033). Instituto Universitario de Oncología del Principado de Asturias is supported by a grant from Fundación Cajastur-Liberbank. Work in the laboratory of P.J.F.M. was funded by the IMDEA Food Institute, by the AECC and Ramón Areces Foundations and by grants from the Spanish Minisitry of Science, Innovation and Universities, co-funded by the European Regional Development Fund (ERDF) (SAF2017-85766-R and RYC-2017-22335). Work in the laboratory of M.S. was funded by the IRB, by "La Caixa" Foundation, and by grants from the Spanish Ministry of Economy co-funded by the European Regional Development Fund (ERDF) (SAF2017-82613-R) and from the European Research Council (ERC-2014-AdG/669622). J.I.S. is supported by NIH grants HL-147070, HL-71643, and AG-49665.

Authors' contributions: J.B.P., C.H.F., I.L.A., and G.M.A. designed the study. J.B.P., C.H.F., I.L.A., L.A.R., P.M.V., C.L.M., I.C., C.P. and P.J.F.M. performed the experiments. G.M.A. did the bioinformatic analysis. J.B.P., I.L.A., C.H.F., L.A.R., P.J.F.M., and G.M.A. analyzed the results. C.H.F., I.L.A., L.A.R., P.J.F.M., M.S., J.I.S., and G.M.A. discussed the significance of the results. J.I.S., M.S., and G.M.A. wrote the article. G.M.A. is the responsible of the integrity of the whole work.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.trsl.2021.01.008.

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