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Over-expression of *Spock2* in mice leads to altered lung alveolar development and worsens lesions induced by hyperoxia

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Running head (60 caractères espaces compris): over expression of *Spock2* and altered lung development

Abstract

SPOCK2 was previously associated with genetic susceptibility to bronchopulmonary dysplasia in a French population of very preterm neonates. Its expression increases during lung development and is increased after exposure of rat pups to hyperoxia as compared to controls bred in room air.

To further investigate the role of *SPOCK2* during lung development, we designed two mouse models, one that uses a specific anti-Spock2 antibody and one that reproduces the hyperoxia-induced *Spock2* expression with a transgenic mouse model resulting in a conditional and lung-targeted over-expression of *Spock2*.

When mice were bred under hyperoxic conditions, treatment with anti-Spock2 antibodies significantly improved alveolarization. Lung over-expression of *Spock2* altered alveolar development in pups bred in room air and worsened hyperoxia-induced lesions. Neither treatment with anti-Spock2 antibody, nor over-expression of *Spock2* were associated to abnormal activation of mmp2. These two models did not alter the expression of known players in alveolar development.

This study brings strong arguments for the deleterious role of *SPOCK2* on lung alveolar development especially after lung injury, suggesting its role in BPD susceptibility. These effects are not mediated by a deregulation in metalloproteases activity and in expression of factors essential to normal alveolarization. The balance between type 1 and 2 epithelial alveolar cells may be involved.

Keywords: lung development, bronchopulmonary dysplasia, Spock2, transgenic model

INTRODUCTION

Bronchopulmonary dysplasia (BPD) is the most common chronic respiratory disease in premature infants but its pathophysiological mechanisms are not completely determined. BPD appears to be a multifactorial disease resulting from the combination of environmental and genetic factors in immature lungs (12). Whereas the nature and the role of environmental factors are well determined, to our knowledge, no study thoroughly investigated the role of genetic factors from the identification of the loci of susceptibility to the function of the encoded protein. By performing the first genome wide association study in a population of very preterm neonates, we previously identified 2 single nucleotide polymorphisms (SNP) in *SPOCK2* significantly associated to BPD defined by the need for supplemental oxygen at 36 weeks post-menstrual age (11). *SPOCK2* (SPARC/osteonectin, CWCV, and Kazal-like domains proteoglycan 2), also known as Testican-2, is a member of the testican group of extracellular chondroitin and heparin sulfate proteoglycans. Its role has mainly been explored in the central nervous system (26, 28). We previously showed that *Spock2* was expressed in the developing rat lung with an increasing mRNA expression very close to the beginning of alveolarization and that its expression was increased in lungs from rat pups exposed to hyperoxia (11). We also showed that *Spock2* was synthesized by lung fibroblasts and epithelial cells and then deposits throughout the extracellular matrix (ECM), including the basement membrane (11). *Spock2* interacts with matrix metalloproteinases (MMP) 14 and 16 (20) that were respectively shown to play a key role in lung development (4, 5) and to be associated to the risk of BPD (10). Thus, *Spock1* and *Spock3* inhibit the proteolysis by MMP14 and MMP16 of pro-MMP2 into its active form. *Spock2* abolishes this inhibition by binding to the COOH terminal domain of *Spock3* through its NH₂-terminal unique domain (20). In the developing lung, MMP2 acts as a major protease during secondary septation.

Nevertheless, the exact role of Spock2 during lung development is not determined and it is not known whether it is mediated by the activation of MMP2.

To better understand the role of Spock2 in alveolar development impairment, we designed two mouse models, one that uses a specific anti-Spock2 antibody and one that reproduces the hyperoxia-induced *Spock2* expression with a transgenic mouse model resulting in a conditional and lung-targeted over-expression of *Spock2*.

MATERIALS AND METHODS

Ethical approval

Ethical approval for all animal experimentation was obtained from the local ethics committee (N° 12-059, ComEth Afssa/ENVA/UPEC, Maisons-Alfort, France). Animals were housed in the transgenic animal facility at the *Institut Henri Mondor de Recherche Biomédicale* (IMRB) and transferred to the *plateforme d'exploration fonctionnelle du petit animal* of the IMRB for experiments.

Antibody model

The aim of this model was to study the effect of the inhibition of Spock2 by a specific antibody on lung development under physiologic and hyperoxic conditions. Wild-type C57Bl6J mouse pups and a goat anti-mouse Spock2 antibody (product # AF2328, R&D systems, Lille, France) were used. First, to determine the dose and the number of injections needed, antibodies' concentrations in mouse lungs were measured following an intra-peritoneal injection of 2 or 5 mg/kg of the antibody. Briefly, P10 mouse pups were treated and then euthanized at 24h, 48h and 72h after the antibody administration by intraperitoneal injection of a mixture of 10% ketamine (Merial, Lyon, France) and 10% xylazine (Bayer, Puteaux, France) in physiological saline. Lungs were extracted, frozen and then homogenized, and total proteins were extracted. Goat IgG concentrations were then measured using the Goat IgG ELISA Kit (Alpha Diagnostic International, San Antonio, Texas, USA). Second, to determine the localization of the antibody in lung parenchyma, immunohistochemistry using the RTU Vectastain PK7800 kit (Vector Laboratories, Burlingame, CA, USA) that detect goat IgG was performed on P10 mouse lungs 6h after an intra-peritoneal injection of either 5mg/kg of anti-Spock2 antibody, 5mg/kg of control goat IgG or PBS. The control goat IgG was used as a control for the anti-Spock2 antibody specificity. Once the dose was determined and the

presence of the specific antibody in the lung parenchyma was confirmed, mouse pups were treated at P5, P8 and P11 by an intra-peritoneal injection of 5mg/kg of the specific antibody diluted in PBS, or an intra-peritoneal injection of 5mg/kg of the control goat IgG, or by an intra-peritoneal injection of PBS alone (negative control). Pups were then euthanized as described above at P14 and the lungs were extracted. Tissues were immediately placed in liquid nitrogen and kept at -80°C until RNA or proteins extractions, or fixed with 10% pre-filtered formalin at a constant hydrostatic pressure of 25 cm H₂O for at least 20 minutes for evaluation of morphometry and immunohistochemistry.

Transgenic model

We designed a Knock-In at Rosa26 locus, with a construction similar to the one that we successfully used for over-expression of the gene FGF18 (9), and that have been extensively described by Whitsett et al (32). For the targeting vector construct, the mouse *Spock2* cDNA was placed under the control of the (teto)7CMV promoter. Polyadenylation sequences from the bovin growth hormone were used to ensure transcript termination. A hygromycin cassette flanked by FRT sites was introduced as a selection marker downstream of the *Spock2* cDNA. Two Homology arms for the Rosa26 Locus were used. The targeting constructs were introduced by electroporation into embryonic stem cells from the 129/SV mouse strain and selected on plates containing hygromycin. Appropriate clones were identified by PCR and confirmed by Southern blot analysis with 5'-3' and an internal probe. A karyotype and the sequencing of the homologous recombinant clones have been done. Stem cells carrying the constructs were injected onto blastocysts from C57BL/6 N mice, to obtain chimeric mice. After germline transmission, the generated *Spock2* KI mice were initially crossed with a germline Flp-deleter murine line to eliminate the hygromycin cassette and then were bred to SP-C-rtTA mice in which the expression of the rtTA activator construct is targeted on

alveolar epithelial type II cells by the promoter of surfactant protein (SF)-C (Figure 1). This provided double transgenic mice that were heterozygous for each transgene, thus permitting to conditionally induce the expression of *Spock2* in the lung. Genotyping was performed on DNA extracted from mouse tails by use of the Charges Switch gDNA kit Mini Tissue kit from Invitrogen (Cergy-Pontoise, France) using adequate primers for PCR in a Biometra thermocycler for SP-C-rtTA, 5'-GACACATATAAGACCTGGTCA-3' (forward) and 5'-AAAATCTTGCCAGRCTTCCCC-3' (reverse) and for (teto)7-*Spock2*, 5'-ACTTGCTCTCCCAAAGTCGCTCTGAG 3' (forward) and 5'-TACCTTTTGATAAGGCTGCAGAAGGA 3' (reverse). *Spock2* over-expression in mice pups was induced by adding doxycycline (1mg/ml) to the drinking water of the mother from P0 to P14.

Hyperoxic model

Exposure to hyperoxia induces alveolar growth disorders in newborn mice. Mice pups and their dams were placed in Plexiglas exposure chambers (Charles River, France) and exposed in parallel to 85% or 21% (room air) FiO₂ from P3 to P14. The oxygen concentration was regularly monitored. Because adult mice have limited resistance to high oxygen, the dams were switched daily between O₂-exposed and room air-exposed litters. The chambers were opened 20 minutes every day to switch the dams and clean the cages. On P14, the pups were euthanized as described above and the lungs were extracted. Tissues were immediately placed in liquid nitrogen and kept at -80°C until RNA or proteins extractions, or fixed with 10% pre-filtered formalin at a constant hydrostatic pressure of 25 cm H₂O for at least 20 minutes for evaluation of morphometry and immunohistochemistry. For all experiments, double transgenic mice were compared to SP-C-rtTA simple transgenic mice that did not over-expressed *Spock2* and were referred as control mice.

RNA isolation, Reverse Transcription and real-time quantitative PCR (RT-qPCR)

Total RNA was extracted from lung tissue using the RNeasy Protect Mini kit (Qiagen, France) and reverse transcribed (RT) into cDNAs using 2 µg of total RNA, Superscript III reverse transcriptase, random hexamer primers (Invitrogen, France) according to the supplier's protocol. Real-time PCR was performed on QuantStudio™ 6 device (Applied Biosystems, France) using initial denaturation (2 min at 95°C), then two-step amplification program (1 s at 95°C followed by 30 s at 60°C) repeated 40 times. Melt curve analysis was used to check that a single specific amplified product was generated. Reaction mixtures consisted of 10 ng cDNA, SYBR Green 2X PCR Master Mix (Applied Biosystems, France) and forward and reverse primers for the various examined transcripts (displayed in Table 1). Real-time quantification was achieved by measuring the increase in fluorescence caused by SYBR Green dye binding to double-stranded DNA at the end of each amplification cycle. Relative expression was determined by using the $\Delta\Delta C_t$ (threshold cycle) method of normalized sample (ΔC_t) in relation to the expression of a calibrator sample used to normalize data from one plate to another, according to the manufacturer's protocol. Each PCR included a no-template control and a sample without reverse transcriptase. All measurements were performed in triplicates. Hprt mRNA was used as reference.

Lung immunohistochemistry: expression of Spock2 in lung tissue

Immunostaining of P14 lung tissue was performed using 5-µm paraffin sections that were deparaffinized with xylene, rehydrated gradually with graded alcohol solutions and washed with deionized water. Antigens were unmasked by boiling for 20 minutes in 10mM citric acid buffer pH 6. After cooling at room temperature, sections were rinsed in phosphate-buffered saline (PBS), pH 7.4 (Sigma) and blocked with 2.5% normal goat serum for 20 minutes at

37°C. Then sections were incubated overnight at 4°C in a humidified chamber with a diluted anti-Spock2 rabbit polyclonal antibody (1:300, bs-11966R, Clinisciences, Nanterre, France). After overnight incubation, sections were washed in PBS and endogenous peroxidase was quenched with 3% H₂O₂ for 30 minutes at room temperature. Sections were then incubated with goat biotinylated secondary antibody (1:300, BA-1000, Vector Laboratories) for 30 minutes, followed by the same time of incubation in the Vectastain ABC reagent (Vector Laboratories) according to the supplier's protocol. After further washing, chromogen 3,3'-diaminobenzidine tetrachloride/nickel-cobalt substrate was applied, yielding a brown colored reaction product, and sections were counterstained with hematoxylin (Hematoxylin QS, Vector Laboratories). Light microscopy images were captured using a digital camera. Quantitative analysis was done with self-written Fiji macros in z-thru projection images of ULP (25). Quantification of protein expression was defined by the surface in relation with the number of nuclei.

Lung morphometry

Methods used in this study have been described in detail previously (9). Briefly, lung fixation was performed by tracheal infusion of 10% pre-filtered formalin at constant 25cm H₂O pressure, and fixed lung volume was measured by fluid displacement. After routine processing and paraffin embedding, 5-µm-thick mediofrontal sections through both lungs were stained with hematoxylin and eosin for morphometry. Alveolar airspace, airways, blood vessels larger than 20 µm in diameter, interstitial tissue volume densities and the alveolar surface density were determined using the point counting and mean linear intercept methods (31). Light microscope fields were quantified at an overall magnification of x330 on 20 fields per animal (10 per lung) with a systematic sampling method from a random starting point. All morphometric analyses were performed by a single observer (MLFM) who was unaware of

group assignment. Alveolar surface density ($S_v(a,p)$) was determined using point counting and mean linear intercept (MLI) methods described by Weibel. Absolute surface area (S_a) per lung was calculated by multiplying surface density by lung volume. Radial alveolar count (RAC) was performed according to Emery's method (7).

Zymography analysis of total gelatinase activity

Lung tissues were homogenized in a buffer (Tris-NaCl-CaCl₂-triton) at pH 7.6 (8). The supernatant was subjected to electrophoresis in 8% (wt/vol) polyacrylamide gels containing 1 mg/ml gelatin with sodium dodecyl sulfate (SDS-PAGE; Sigma-Aldrich, Saint-Louis, USA), under non reducing conditions. After electrophoresis, the gels were washed in Triton X-100 2.5% for 1 h, rinsed briefly, and incubated at 37°C for 72 h in buffer containing 100 mM TrisHCl, pH 7.40, and 10 mM CaCl₂. The gels were then stained with Coomassie brilliant blue R250 and restained in a solution of 7.5% acetic acid and 5% methanol. Zones of enzymatic activity were indicated by negative staining: areas of proteolysis were seen as clear bands against a blue background. Enzyme activities in the gel were quantitated using G:Box (Syngene, Synoptics, Cambridge, UK). Relative expression was determined by the method for normalized samples relative to a calibrator sample. Results were expressed as arbitrary units (AU) per 65h per 30 µg of Bactine in the lung tissue.

Statistical analyses

Data were expressed as medians with their interquartile range. Multiple groups' comparisons were performed by Kruskal-Wallis analysis, and two-group comparisons were performed by Mann-Whitney U test using GraphPad Prism software (GraphPad software INC, San Diego, CA). A p value<0.05 was considered significant.

RESULTS

Antibody model

Development of the model

Twenty-four hours after an intra-peritoneal injection of 5 mg/kg of anti-Spock2 antibody in P10 mouse pups, concentration of goat IgG in lungs was measured at 121 ng/mg of total protein content. Residual concentrations were measured at 46.1 and 47 ng/mg of total protein content 48h and 72h after the injection respectively (supplemental Figure 1). For the 2 mg/kg dose, concentration at 24h was very low (54.4 ng/mg). Therefore we chose the dose of 5 mg/kg for the following experiments.

Six hours after an intra-peritoneal injection of either anti-Spock antibody, control goat IgG or PBS in P10 mouse pups, lungs' immunohistochemistry revealed that the anti-Spock2 antibody was fixed in lung parenchyma whereas control goat IgG remained exclusively in the intra-vascular bed (Figure 2). No signal was detected after PBS injection.

Treatment with anti-Spock2 antibodies partially protects from hyperoxic injury

As hyperoxia exposure led to an over-expression of *Spock2*, we assessed in wild-type animals whether inhibition of *Spock2* by a specific antibody will have an effect on alveolarization, either in room air or after hyperoxia exposure. In control animals injected with PBS, hyperoxia exposure led to alveolar disruption with larger and fewer alveoli as shown by a increased MLI that reflects a diminution in septa's number and a decreased in alveolar surface (S_a and $S_{v(a,p)}$) (Figure 3). Treatment with anti-Spock2 antibodies did not modify morphometric parameters when mice were bred in room air (Figure 3). When mice were bred under hyperoxic conditions, treatment with anti-Spock2 antibodies significantly improved alveolarization with a decreased MLI ($p=0.024$), and increased values of $S_{v(a,p)}$ ($p=0.015$) and S_a ($p=0.026$) as compared to animals exposed to hyperoxia injected with PBS (Figure 3).

Nevertheless, treatment with anti-Spock2 antibodies under hyperoxic conditions did not completely restore a normal alveolar development as suggested by the significant differences in morphometric analyses between mice bred in room air without treatment and mice bred under hyperoxic conditions that were injected with anti-Spock2 specific antibodies (Figure 3). Treatment with the control goat IgG did not alter morphometric parameters in comparison with injection with PBS, not in room air neither after hyperoxia exposure (Figure 3).

Effect of anti-Spock2 antibodies on Mmp2 activation

As Spock2 was shown to abrogate the inhibition of mmp14 and 16 by Spock1 and Spock3 for the proteolysis of pro-mmp2 in its active form *in vitro* (20), we studied the activation of mmp2 after treatment with anti-Spock2 antibodies in room air (n=8) and under hyperoxic conditions (n=6). As previously reported in the literature (13, 22), in control mice (injected with PBS), hyperoxia exposure led to a significant diminution of the activated fraction of mmp2 (21.8 % in room air *versus* 4.9 % after hyperoxic exposure, $p=0.0002$ Figure 4). Anti-Spock2 antibody injection led to a decrease of the activated fraction in room air (21.8 % in animals injected with PBS *versus* 12.6 % in animals injected with anti-Spock2 antibody, $p=0.0027$ Figure 4). There were no significant differences for the activated fraction of Mmp2 between animals injected with PBS and animals injected with the antibody under hyperoxic conditions.

Anti-Spock2 antibodies does not alter the expression of main players in alveolar development

Expression of known players in alveolar development was quantified by RT-qPCR in the different experimental conditions. Expression of those genes was significantly decreased in control animals after exposure to hyperoxia as compared to control animals bred in room air (Figure 5). Hyperoxia had the same effect in animals treated with the anti-Spock2 antibody, as

compared to animals treated with the anti-Spock2 antibody bred in room air (Figure 5). Nevertheless, no significant difference was observed between control animals and animals treated with the anti-Spock2 antibody, either in room air or after hyperoxia exposure for any of the genes that were studied (Figure 5).

Transgenic model

Spock2 expression

Spock2 expression was quantified in double-transgenic mice (n=9) and controls (n=5) by RT-qPCR at P14. *Spock2* expression was significantly increased in double transgenic mice (n=9) as compared to controls (n=5) with a median 30-fold increase (p=0,001). At the protein level, Spock2 expression was quantified by immunohistochemistry at P14. Immunostaining was significantly more intense in double transgenic mice (n=5) than in controls (n=7) (p=0,048) (Figure 6).

Lung over-expression of Spock2 altered alveolar development in pups bred in room air and worsened hyperoxia-induced lesions

Morphology of lung parenchyma was analyzed at P14 from control and transgenic mice bred either in room air or under hyperoxic conditions. In room air, *Spock2* over-expression induced significant alterations in alveolarization with an increased mean linear intercept (MLI) (156 versus 137, p=0.018), a decreased alveolar area density ($S_{v(a,p)}$) (276 vs 319, p=0.011), and a decreased absolute area of airspaces (S_a) (52 vs 82, p=0.018) in double transgenic mice (n=5) as compared to controls (n=14) (Figure 7). These alterations in morphometric parameters reflect a disruption in alveolar development with fewer and larger alveoli as shown in panel 1.B as compared to panel 1.A in Figure 7. As expected in control animals (24), hyperoxia exposure altered alveolar development with an increased MLI (173.5 vs 137, p<0.0001), and

decreased values of $S_{v(a,p)}$ (241 vs 319, $p<0.0001$), specific alveolar area density ($S_{v(a,p)spec}$) (3529 vs 5585, $p<0.0001$), S_a (66.5 vs 82, $p=0.002$), specific absolute surface area of airspaces ($S_{a spec}$) (949 vs 1471, $p=0.0004$) and radial alveolar count (RAC) (7.65 vs 9.64 $p=0.04$) as compared to controls in room air (Figure 7 panel 2 and panel 1.C *versus* panel 1.A). After exposure to hyperoxia, double transgenic mice ($n=6$) displayed aggravated alveolar lesions as compared to control mice ($n=6$), with an increased MLI (213 vs 173.5 $p=0.026$), and decreased values of $S_{v(a,p)}$ (204 vs 241 $p=0.026$) and $S_{v(a,p) spec}$ (2965 vs 3529 $p=0.041$) (Figure 7 panel 2 and panel 1.D *versus* panel 1.C).

Alveolar lesions induced by Spock2 over-expression are not associated to an increased activation of Mmp2

We studied the activation of mmp2 in our models ($n=6$ per group). As previously reported in the literature (13, 22), in control mice, hyperoxia exposure led to a significant diminution of the activated fraction of mmp2 (27.1% in room air *versus* 12.6% after hyperoxic exposure, $p=0.002$, Figure 8). Nevertheless, there were no difference for the activated fraction of mmp2 between double transgenic mice and control mice either in room air or after exposure to hyperoxia (21.5% vs 27.1% in room air, $p=0.48$ and 14.3% vs 12.6% under hyperoxic conditions, $p=0.24$, Figure 8).

SPOCK2 over-expression does not alter the expression of main players in alveolar development

To further investigate the mechanisms by which over-expression of *Spock2* alters alveolar development, expression of known players in alveolar development was quantified by RT-qPCR in the different experimental conditions. For control animals, expression of *Fgfr4* and

Pdgfr were significantly lower in animals bred under hyperoxic conditions as compared to animals bred in room air. For transgenic mice, expression of *Vegfa*, *Fgfr4*, *Pdgfr* and *Tgfb* were significantly lower in animals bred in hyperoxic conditions. Nevertheless, no significant difference was observed between double transgenic and control mice either in room air or after hyperoxia exposure for any of the genes that were studied (Figure 9).

DISCUSSION

We previously identified 2 SNP in the *SPOCK2* gene that were associated with the development of BPD in a large French cohort of very preterm neonates (11). Our results were independently replicated in a Finnish population (11). Here we report a significant effect of *Spock2* over-expression on alveolar development in a mouse transgenic model that conditionally over-expressed *Spock2* during alveolarization in lungs of mouse pups. Over-expression of *Spock2* led to alterations of morphometric parameters and worsened the lesions that were induced by hyperoxia. Inhibition of the Spock2 protein by a specific antibody partially protects from the hyperoxic aggression. Altogether, these results are in favor of a deleterious effect of *Spock2* over-expression on alveolar development especially in a situation of neonatal lung injury and suggest a role of Spock2 in hyperoxia induced lesions. These results are of particular interest in the setting of BPD where hyperoxic aggression is known to play an important role on its subsequent development in preterm babies (2, 16, 17).

To our knowledge, this work represents the first study in the field of BPD starting from the selection of a locus in a genome-wide association study in humans to the demonstration of the role of the corresponding protein during normal and altered lung development in mice. Four other genome-wide association studies were published (3, 19, 27, 29) and only the study by Ambalvanan et al (3) investigated the expression of the locus that was selected (miR-219)

during lung development and after hyperoxia exposure in mice. Three exome sequencing studies identified many loci with data of quantitative mRNA expression of the corresponding genes coming from large transcriptomic data sets from human or mice lungs but without any further functional studies on animal models (6, 18, 23).

SPOCK2, also known as Testican-2, is a member of the testican group of extracellular chondroitin and heparin sulfate proteoglycans that includes 2 others proteins namely Testican-1 and Testican-3. Testican family's functions were especially studied in relation to the role and over-expression of MMPs in various human malignancies (21). Indeed, it was first shown that SPOCK1 and 3 inhibit pro-MMP-2 processing mediated by MMP14 and MMP16 and that transfection of *SPOCK3* in glioma cells suppressed their invasive growth in collagen gel (21). Further studies demonstrated that SPOCK2 abolishes the inhibition of MMP14 and 16 by SPOCK1 and 3 thus promoting ECM degradation and cell migration and invasion (20). As MMP2, 14 and 16 were shown to play a role during lung development (4, 5, 10, 15), we hypothesized that the role of SPOCK2 in alveolar development could be linked to MMP2 activation. Nevertheless, the hypothesis was not confirmed in our model as there was no significant difference in MMP2 activation between control and transgenic mice either in animals bred in room air or after hyperoxia exposure.

Spock2 over-expression did not alter the expression of several genes that were selected for their roles in alveolar development or the inflammatory response that is triggered by environmental aggressions in the setting of BPD.

Therefore, our results did not bring the mechanism by which SPOCK2 could have a deleterious effect on alveolarization in the setting of a hyperoxic aggression. It appears that it is neither mediated by MMPs activities and ECM remodeling, nor by the main players involved in alveolar development. A recent study identified SPOCK2 as a marker of the early transdifferentiation from alveolar type 2 (AT2) to alveolar type 1 (AT1) epithelial cells (14).

In a whole genome analysis, SPOCK2 was found to be rapidly highly expressed when AT2 cells were cultured on collagen (that promotes transdifferentiation into AT1) whereas its level of expression did not change when AT2 cells were cultured on Matrigel (that retarded transdifferentiation) (14). Furthermore, AT2 cells act as AT1 cells progenitors during development (1) but also after lung injury (30). When considering SPOCK2 as a marker of AT1 cells, its over-expression in a setting of lung injury during development could impair the balance between AT2 and AT1 cells and prevent AT2 cells from their role of progenitors and actors during secondary septation and repairs after epithelial injury such as hyperoxia. In the specific setting of BPD that results in part from aggressions occurring in the developing lungs, the over-expression of SPOCK2 could be associated with a preferred transdifferentiation from AT2 into AT1 cells. This imbalance in favor of AT1 cells would limit the capacity of repairs and regeneration in the lungs and participate to BPD development.

In conclusion, by performing morphometric analyses of transgenic and control mice lungs, we bring strong arguments for the deleterious role of SPOCK2 on lung alveolar development especially after lung injury, suggesting its role in BPD susceptibility. This effect could be mediated by the imbalance between AT2 and AT1 cells with a lack of sufficient AT2 cells to pursue cell proliferation and repair that are necessary to secondary septation and formation of the definitive alveoli. This hypothesis will need further studies to be confirmed.

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Table 1: Sequence of primers used for RT-qPCR determinations

Gene Name	Forward Primer (5'-3')	Reverse Primer (5'-3')	Amplicon (pb)
Hprt	GTTAAGCAGTACAGCCCCAAAATG	TCAAGGGCATATCCAACAACAAAC	134
Spock2	ATCAGCCGCAAGAAGTTGGA	CAGAGCTGTAGGTATGGCCG	139
Fgfr3	GGAGTTCCACTGCAAGGTG	CCTCTAGCTCCTTGTCTGGTG	155
Fgfr4	CTGCTTTGGGCAAGTGGTTC	TGCCAAATCCTTGTCTGGAGG	118
Fgf18	TGAACACGCACTCCTTGCTAGT	GAATTCTACCTGTGTATGAACCGAAA	76
VegfA	TCTCCCAGATCGGTGACAGT	GGCAGAGCTGAGTGTTAGCA	71
Pdgfr	GAGCTTTGAGCGACGCC	GACCAGAAAGACCTGGTGGGA	70
Tgf β 1	GCTAATGGTGGACCGCAAC	ACTGTGTGAGATGTCCTTTGGTTTTTC	58

Figure legends:

Figure 1: Transgenic constructs used for conditional expression of Spock2

Figure 2: Immunostaining of goat IgG in mouse lung tissue 6h after an intra-peritoneal injection of (A) anti-Spock2 antibody, (B) control goat IgG and (C) PBS. A: immunostaining after one injection of anti-Spock2 antibody shows a diffuse staining in alveolar septa (arrow and inset). B: immunostaining after one injection of control goat IgG shows a staining limited to vessels (arrow). C: no staining was observed after one injection of PBS. Magnification: x 400.

Figure 3: Effect of treatment with anti-Spock2 antibodies in control mice bred in room air and after hyperoxia exposure. Panel A. Intra-peritoneal injection of a specific antibody that targets Spock2 partially rescued alveolar lesions induced by hyperoxia exposure, whereas in panel B. injection with the control goat IgG did not alter morphometric parameters either in room air or after hyperoxia exposure Room air injected with PBS (RA/PBS), room air injected with the anti-Spock2 antibody (RA/Ab), hyperoxia exposure injected with PBS (O2/PBS), and hyperoxia exposure injected with the anti-Spock2 antibody (O2/Ab), room air injected with the control goat IgG (RA/IgGc), hyperoxia exposure injected with the control goat (O2/IgGc) *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Figure 4: Zymographic analyses of Mmp2 activation in the antibody model. Activation of Mmp2 was explored by measuring the activated fraction of Mmp2 in the different conditions of the antibody model *ie* room air injected with PBS (RA/PBS), room air injected with the antibody (RA/Ab), hyperoxia exposure injected with PBS (O2/PBS), and hyperoxia exposure injected with the antibody (O2/Ab). **: $p < 0.01$; ***: $p < 0.001$; ****: $p < 0.0001$.

Figure 5: mRNA expression pattern of different genes involved in alveolar development in the antibody model. Although hyperoxia exposure induced a decrease in gene expression for

Fgfr3, Fgfr4, Fgf18, Vegfa, Pdgfr and Tgf β , no significant difference was observed between control animals and animals treated with the anti-Spock2 antibody, either in room air or after hyperoxia exposure. *:p<0.05 ; **:p<0.01 ; ***:p<0.001 ; ****:p<0.0001

Figure 6: Expression of Spock2 in control and double transgenic mice. A. and B. Immunostaining (brown) of Spock2 in control and double transgenic mice over-expressing Spock2. A: Control mouse. B: Double transgenic mouse. Immunostaining was more intense in double transgenic animals (B) than in controls (A). Quantitative analysis (C) confirmed a significant increase in double transgenic mice as compared to controls. Magnification x 400. *: p<0.05

Figure 7: Morphometric parameters in control and double transgenic mice bred in room air or under hyperoxic conditions. 1: histologic sections of control (A and C) and transgenic (B and D) pups' lungs at P14 after exposition to air (A and B) or oxygen (85%) (C and D). 2: morphometric analyses comparing genotypes (control (Ctrl) or double transgenic (KI)) under the 2 different experimental conditions (room air (RA) or hyperoxia (O₂)) showing altered alveolarization in KI as compared to control animals both in room air and after exposure to hyperoxia. Magnification x 100. *:p<0.05 ; **:p<0.01 ; ***:p<0.001 ; ****:p<0.0001

Figure 8: Zymographic analyses of MMP2 activation in the experimental groups. As shown by quantitative analysis exposure to hyperoxia induced a decrease in the activation fraction of MMP2 for both genotypes as compared to room air, but over-expression of SPOCK2 did not modify the activation of MMP2. *:p<0.05 ; **:p<0.01

Figure 9: mRNA expression pattern of different genes involved in alveolar development in the different experimental conditions. Although hyperoxia exposure induced a decrease in gene expression for Fgfr4, Vegfa, Pdgfr and Tgf β , no significant difference was observed between

double transgenic and control mice either in room air or after hyperoxia exposure for any of the genes that were studied. *:p<0.05 ; **:p<0.01 ; ***:p<0.001

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

AH, SG, MDC and CD conceived and designed research; AH, MLF, SG and MDC performed experiments; AH, MLF, SG and CD analyzed data; AH, MLF, SG and CD interpreted results of experiments; AH and MLF prepared figures; AH drafted manuscript; AH, MLF and CD edited and revised manuscript; AH, MLF, SG, MDC and CD approved final version of manuscript.

Target construct

Wild Rosa26 allele

ATG



Transgene



*Mutated allele
following FLP
Recombinase*



Spock2 hemizygous

X

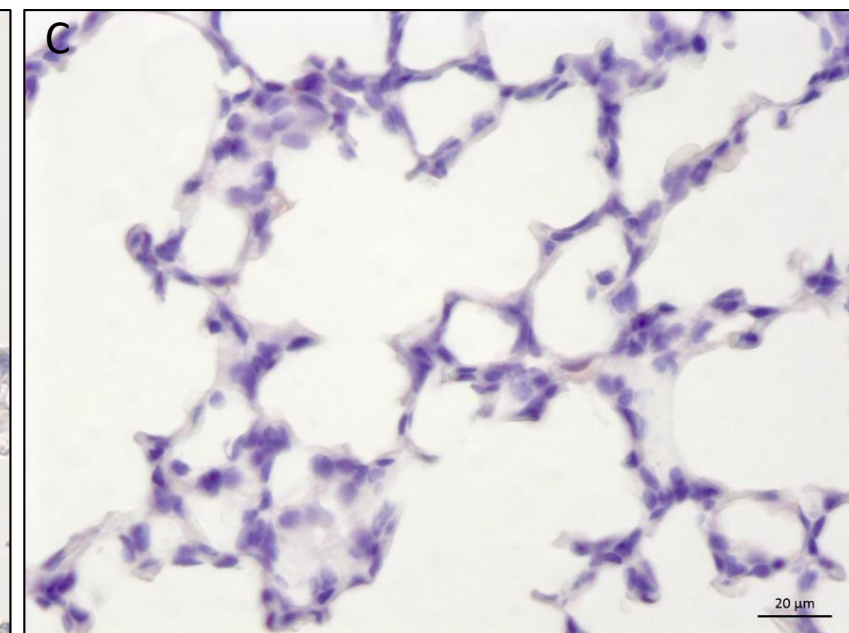
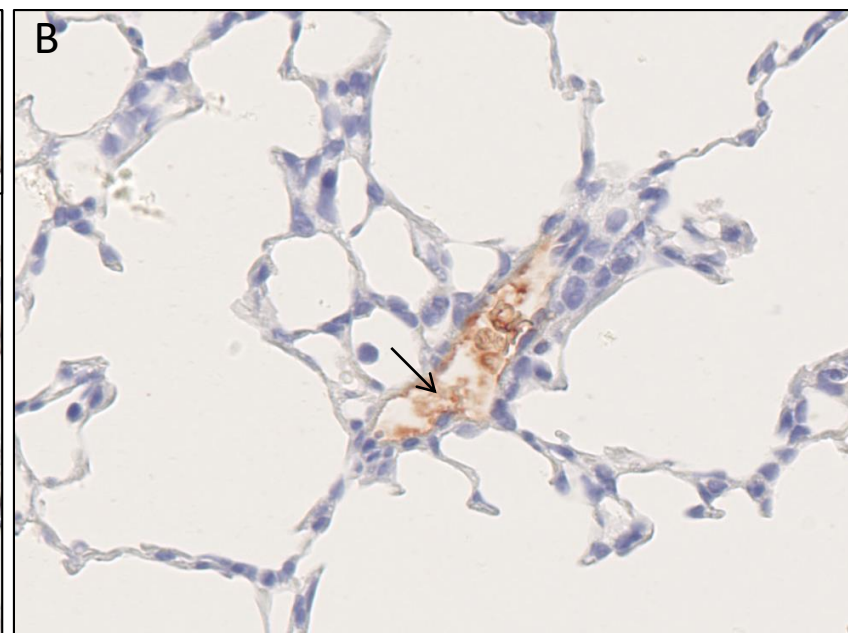
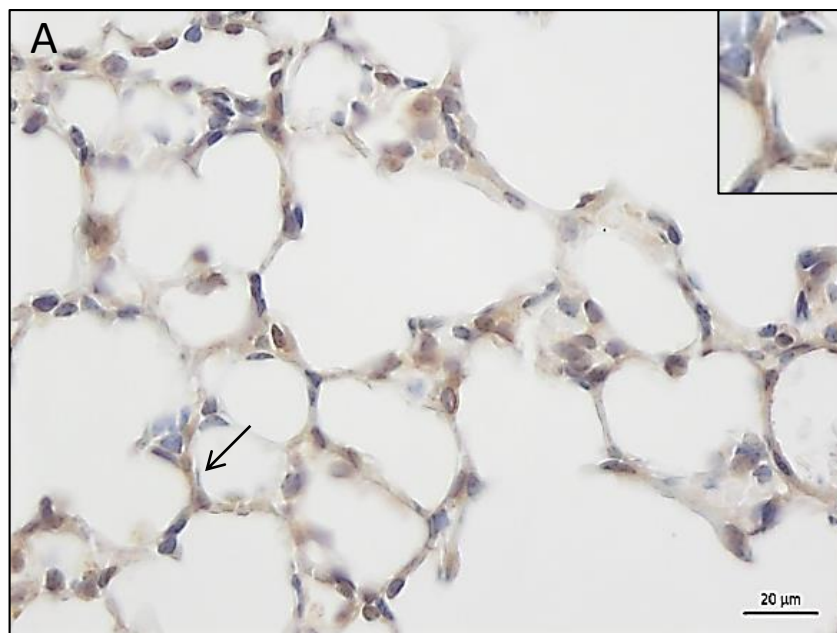


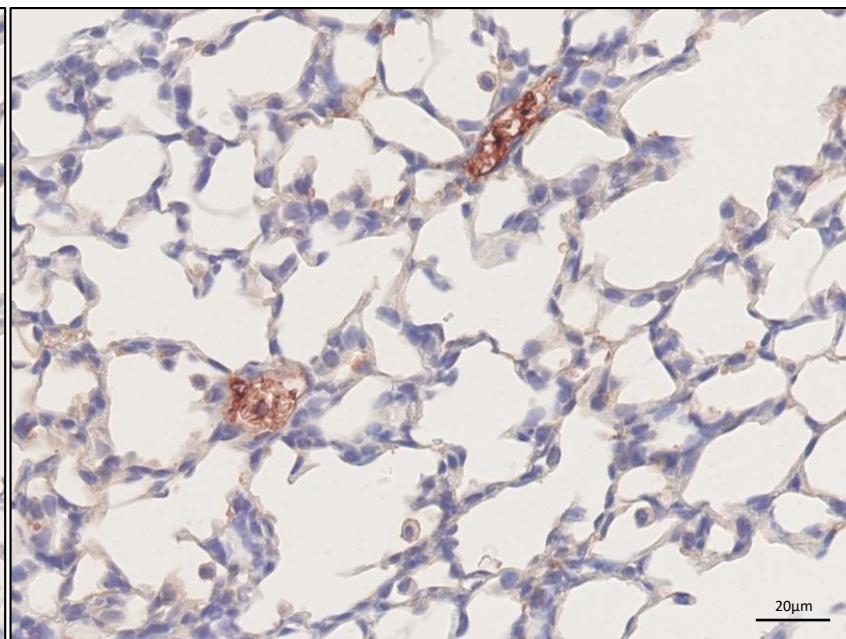
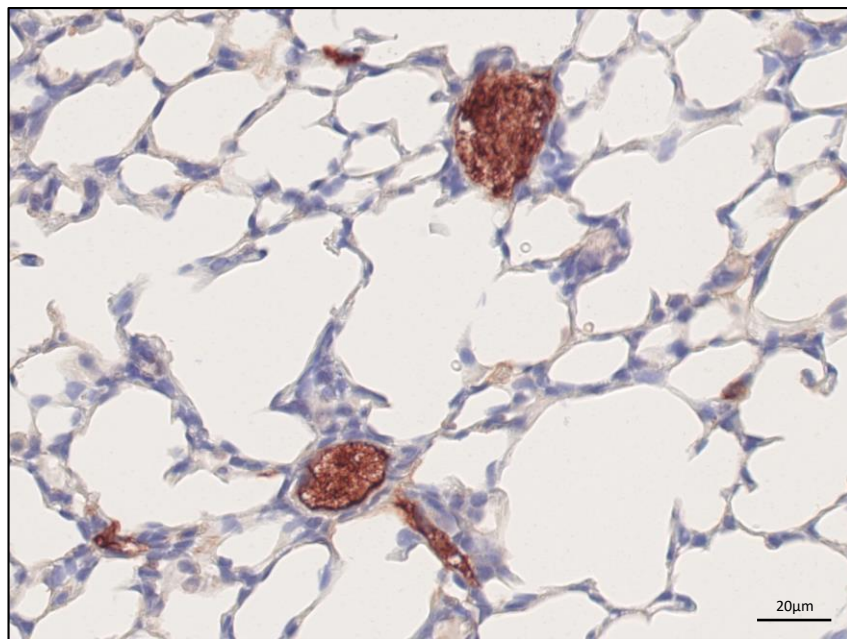
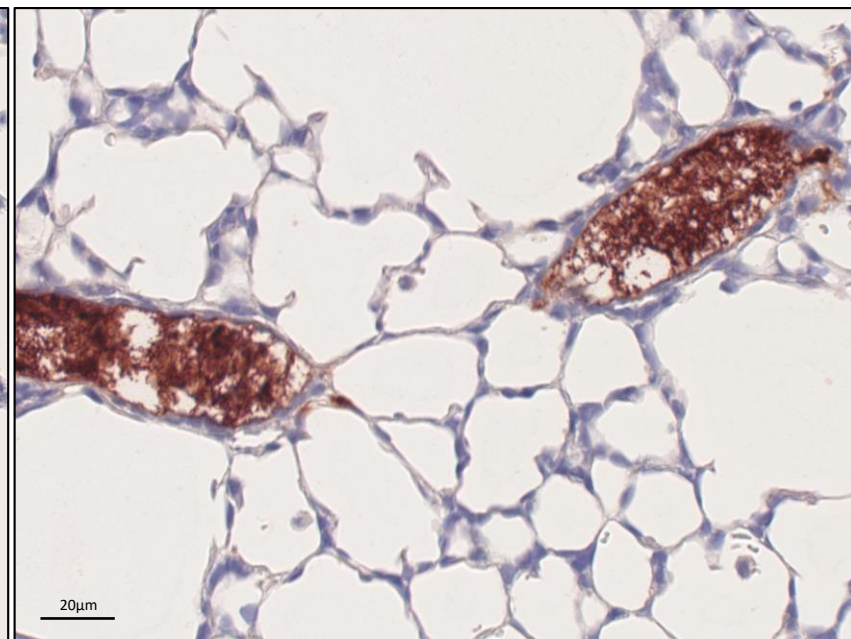
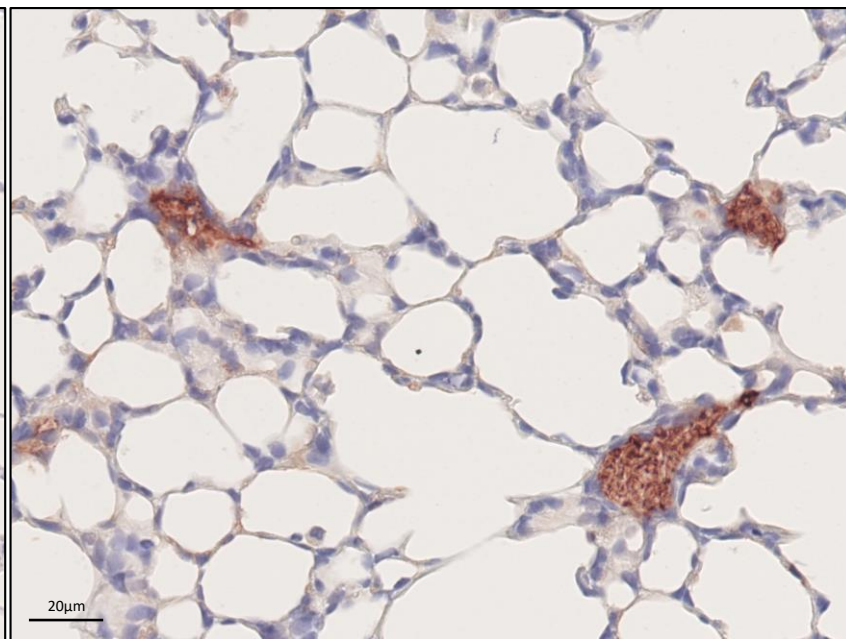
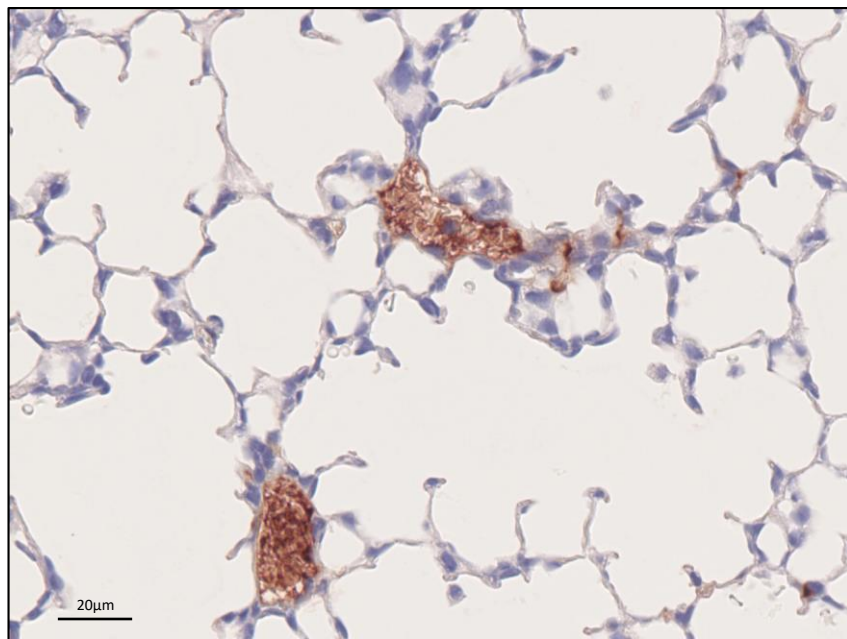
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Spock2 hemizygous**

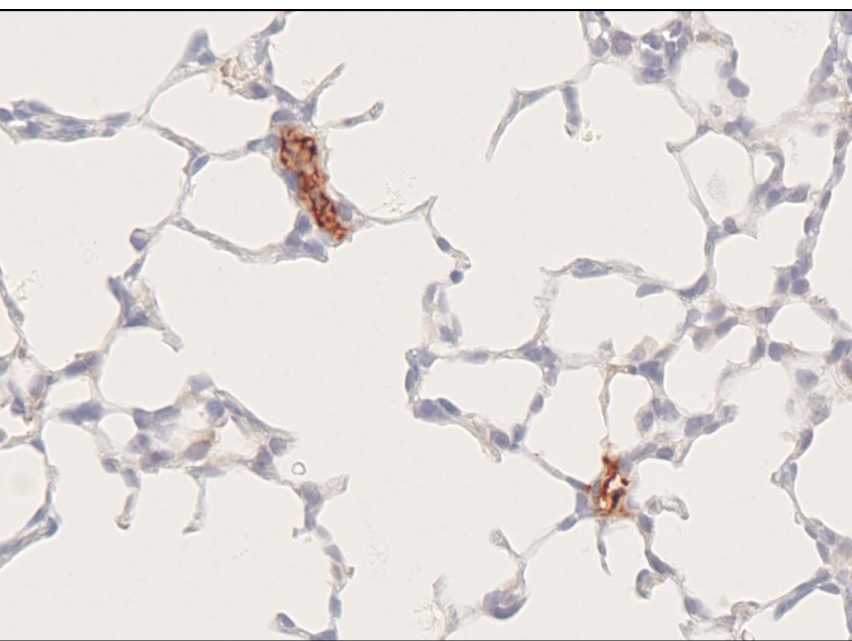
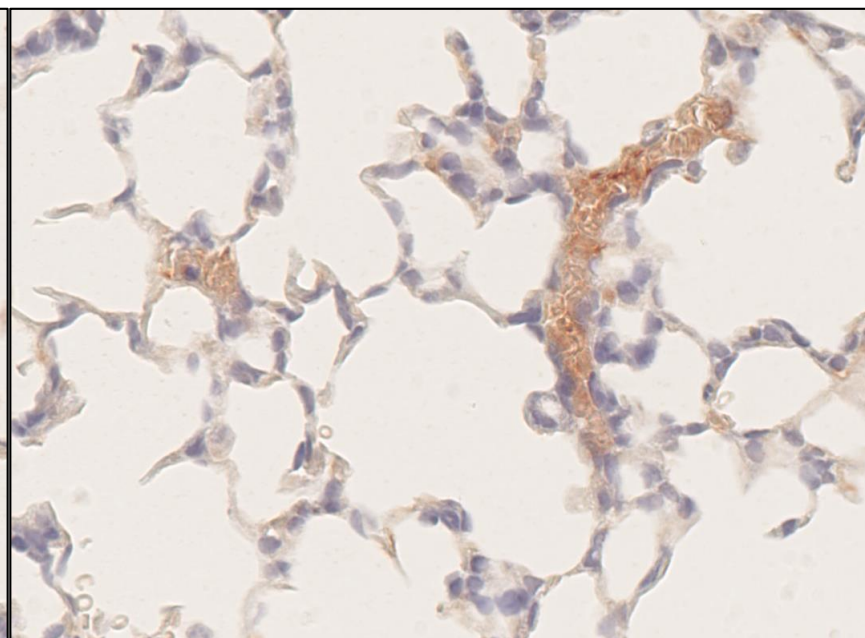
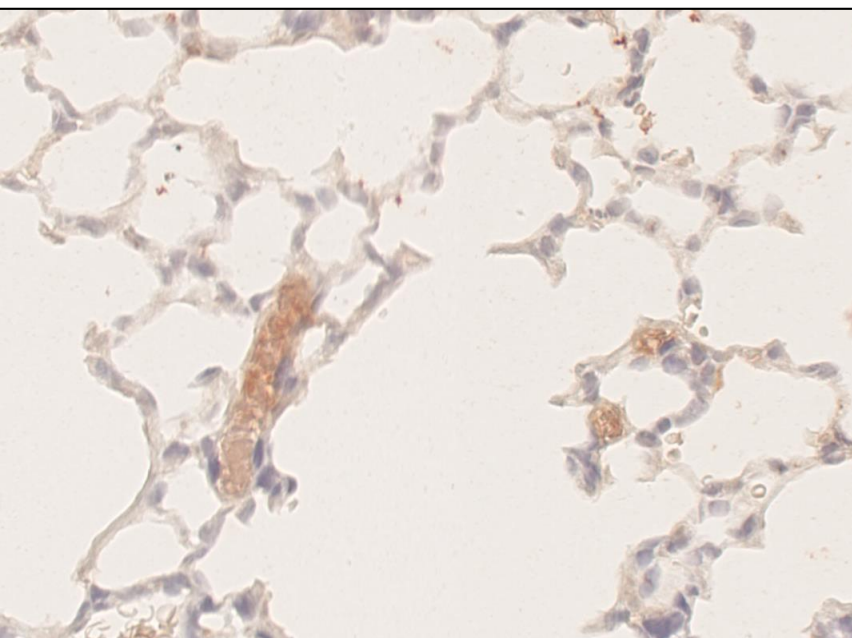
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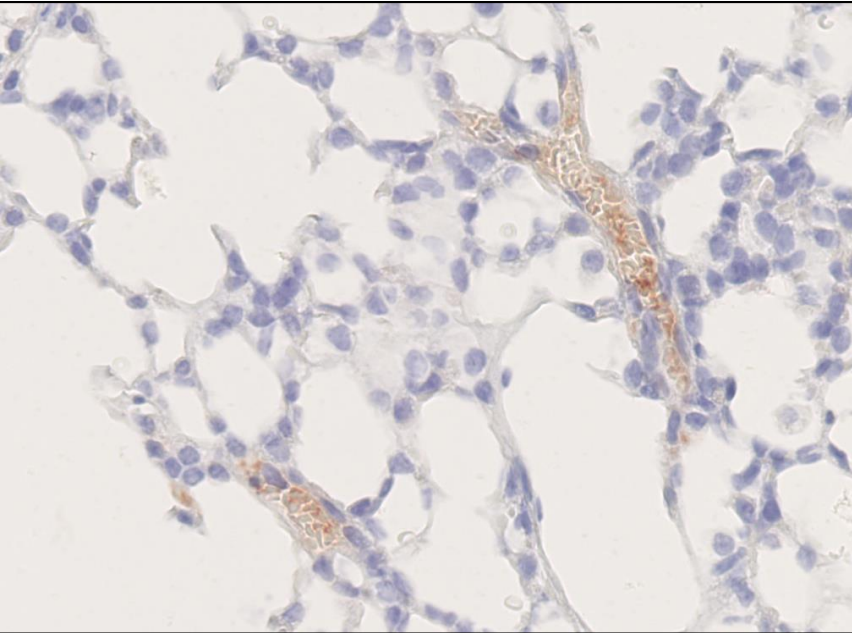
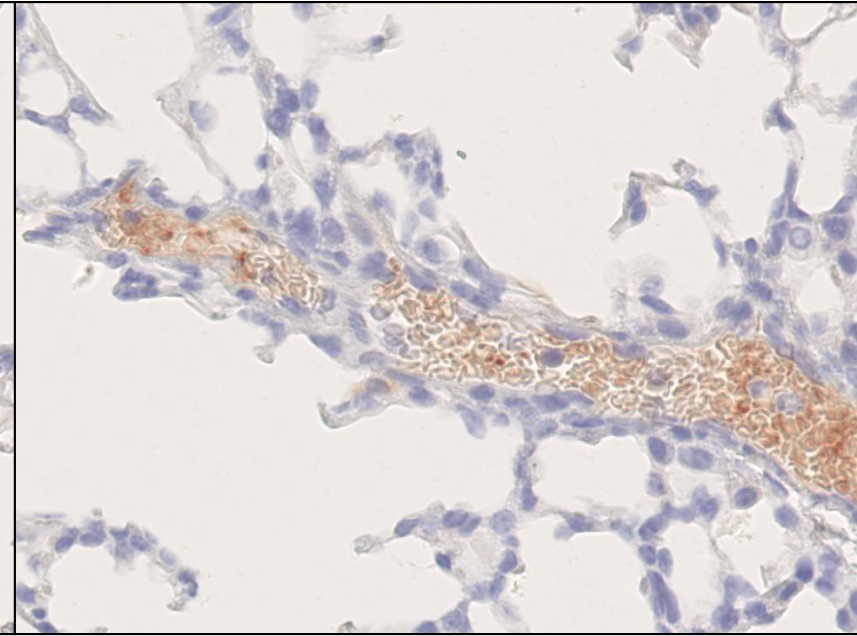
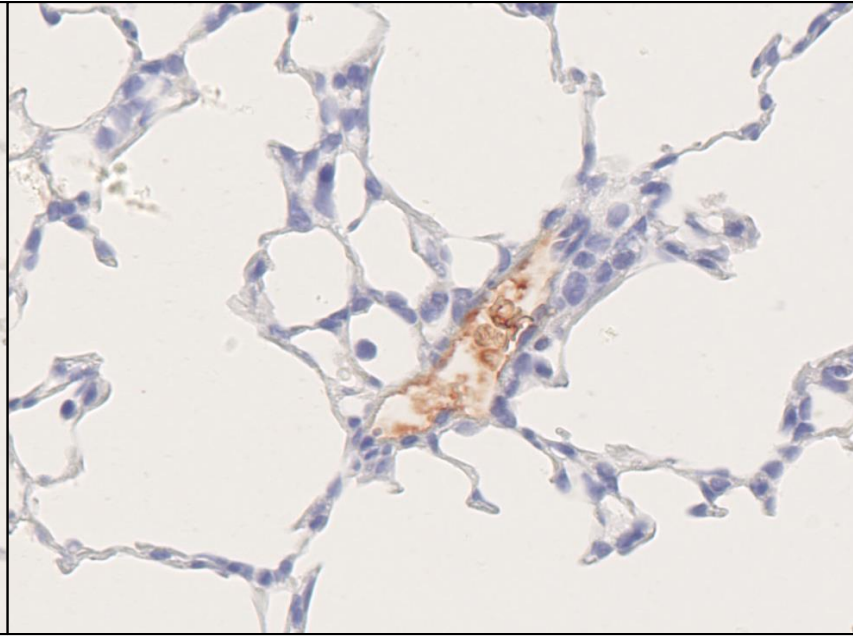
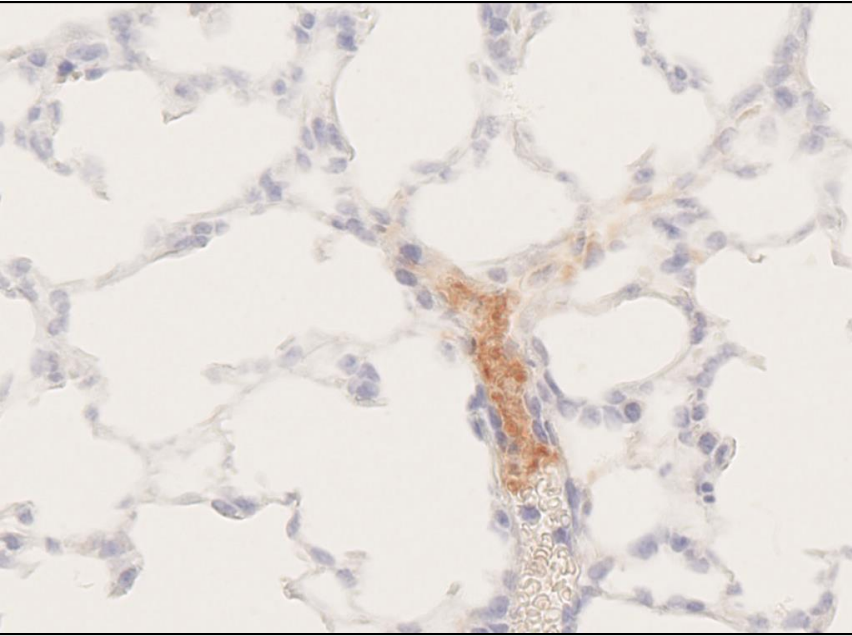


rtTA hemizygous



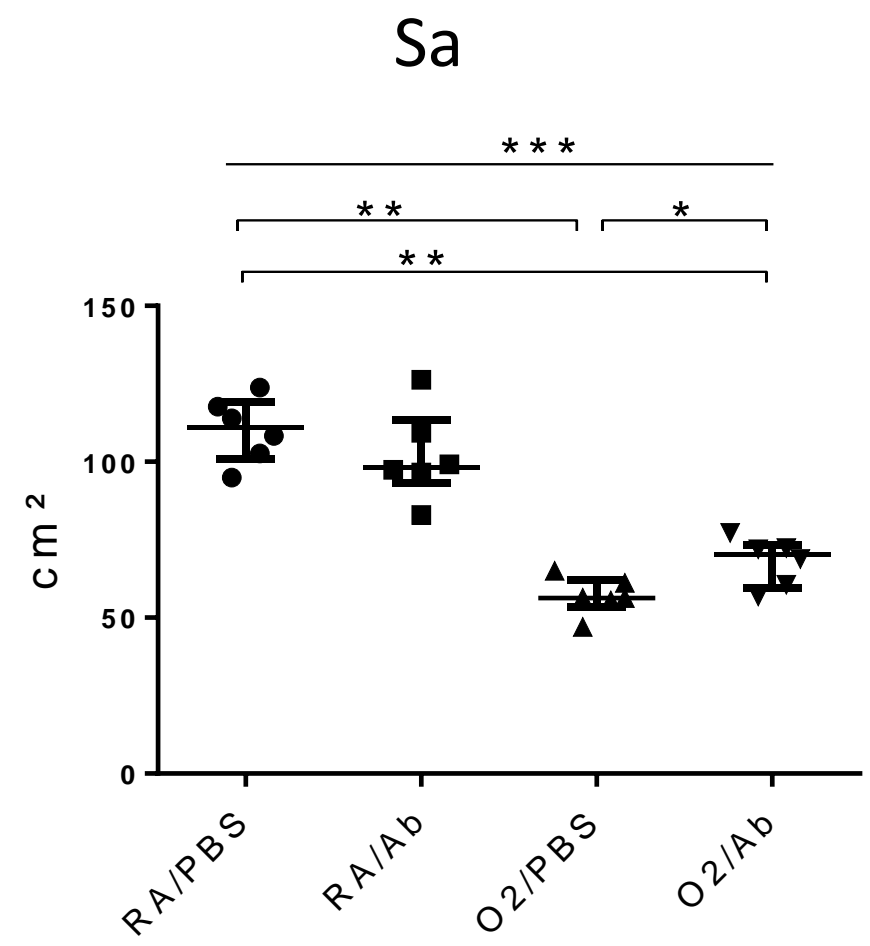
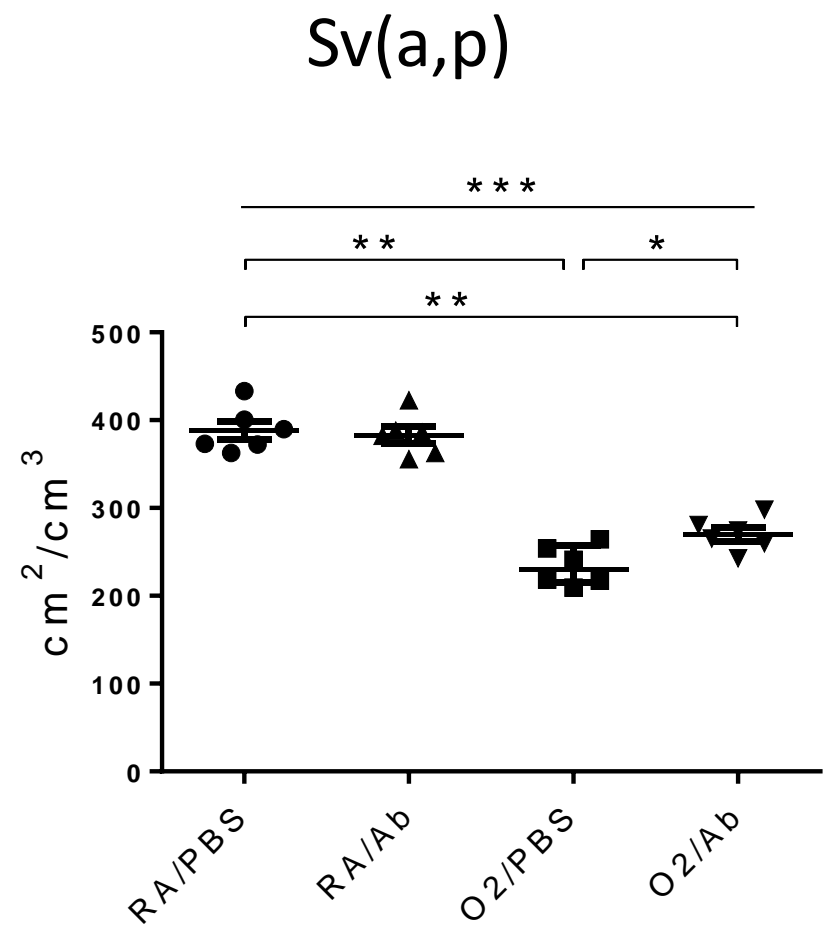
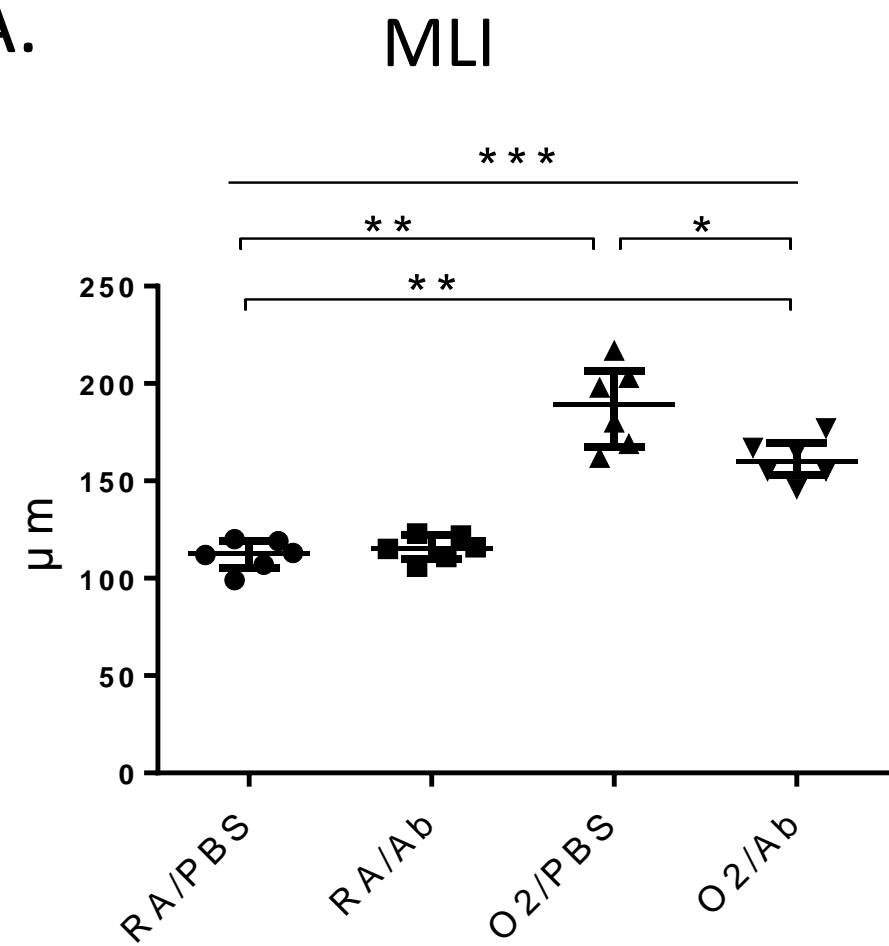




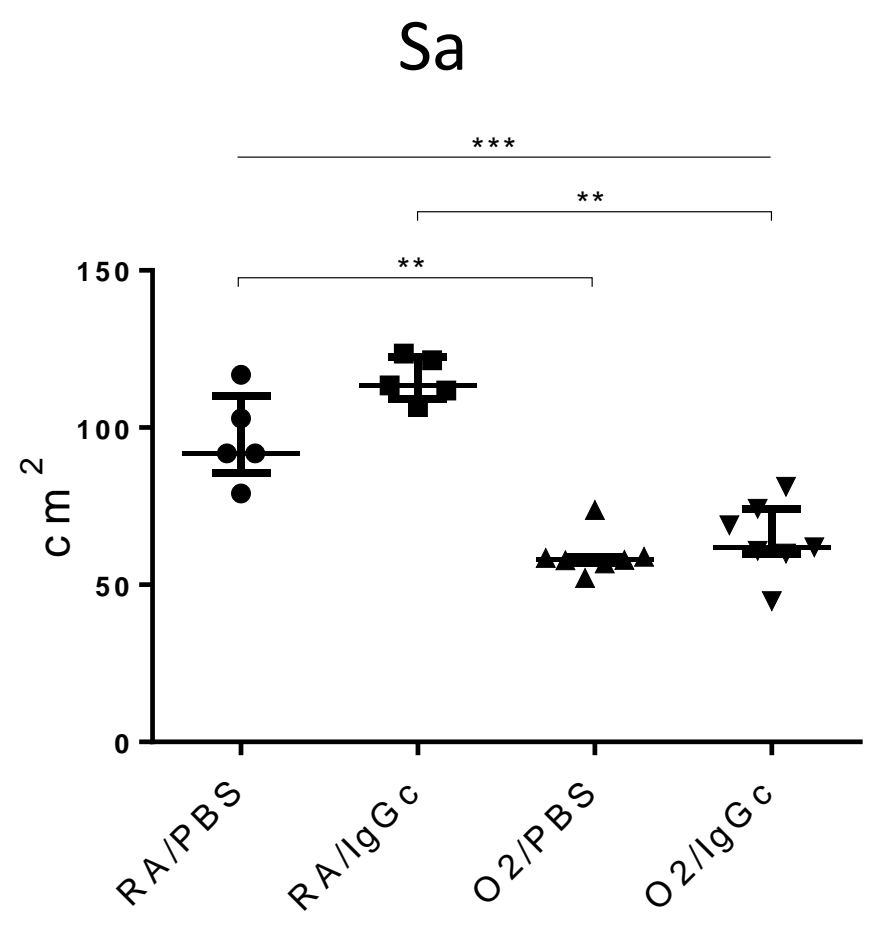
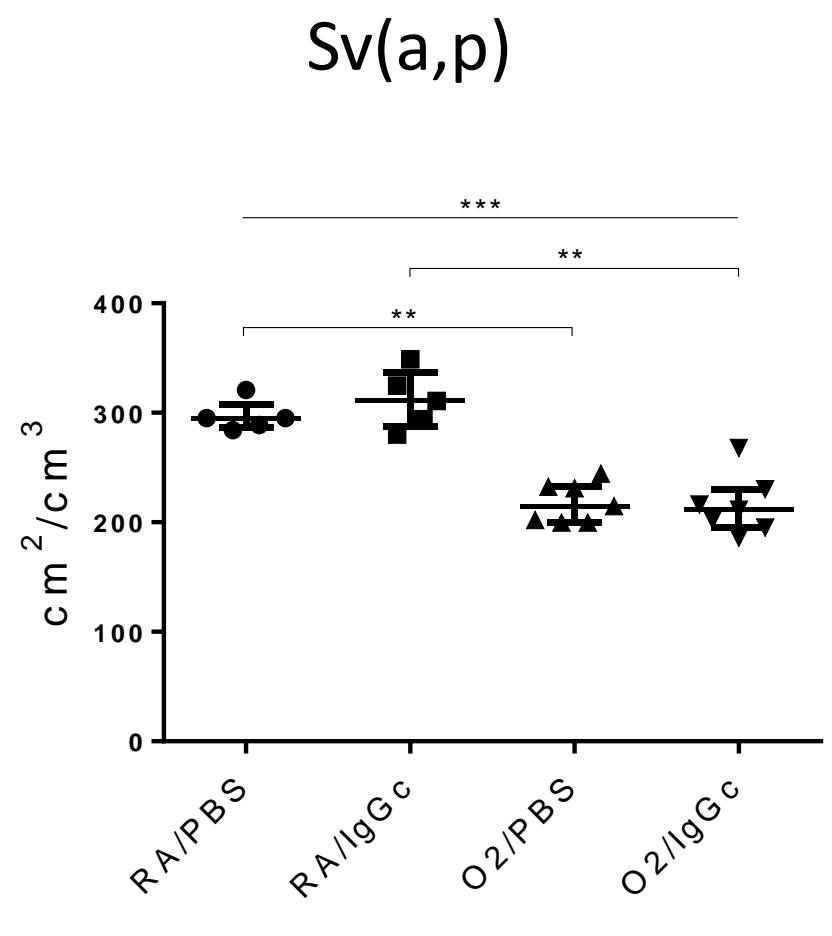
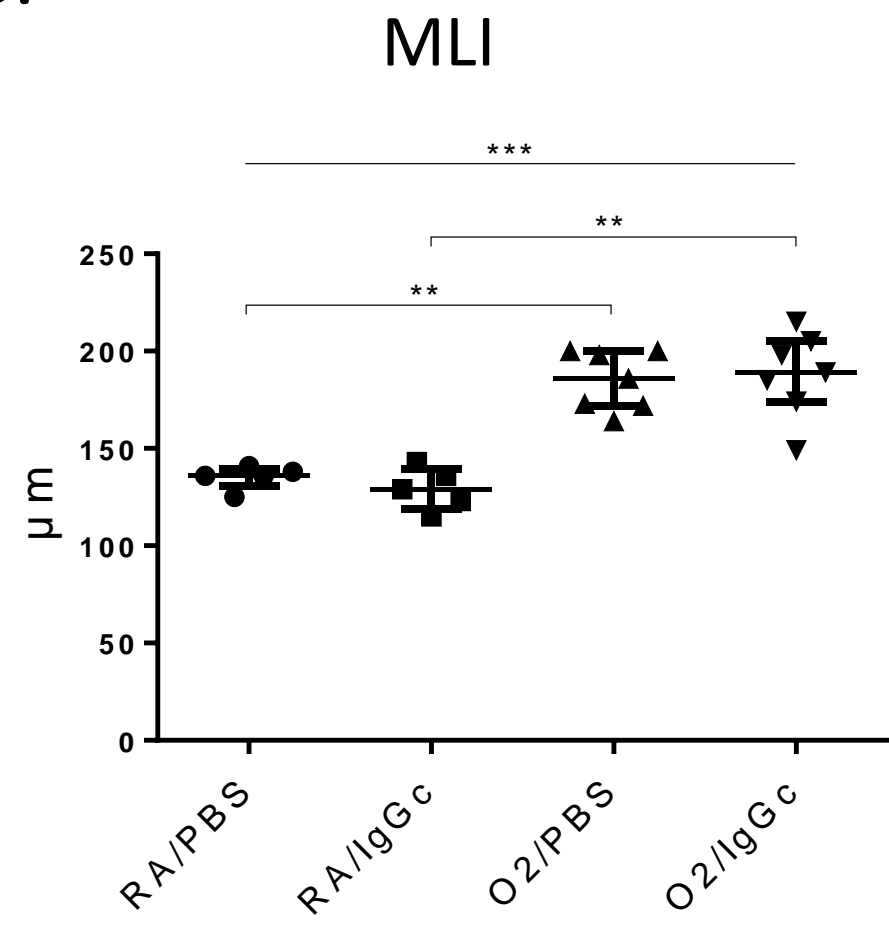


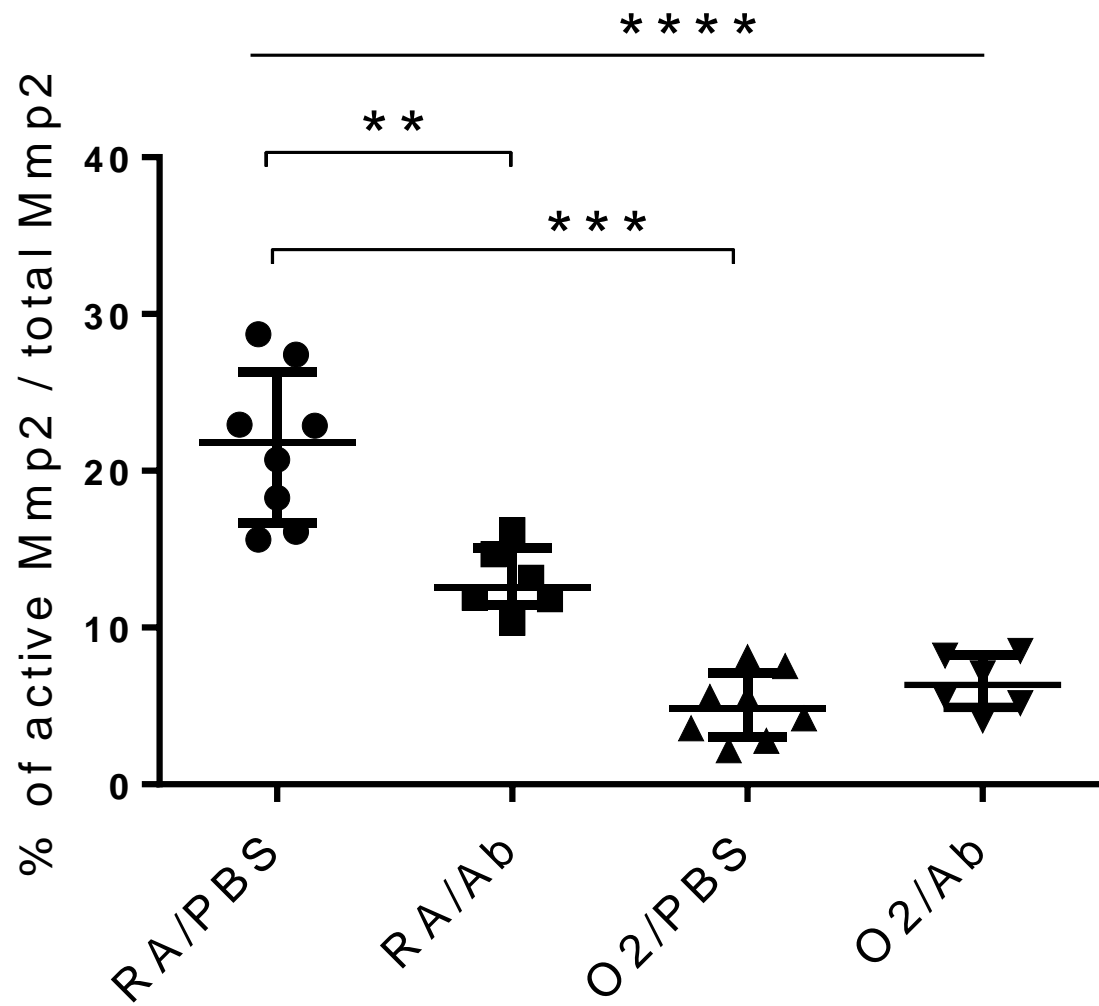


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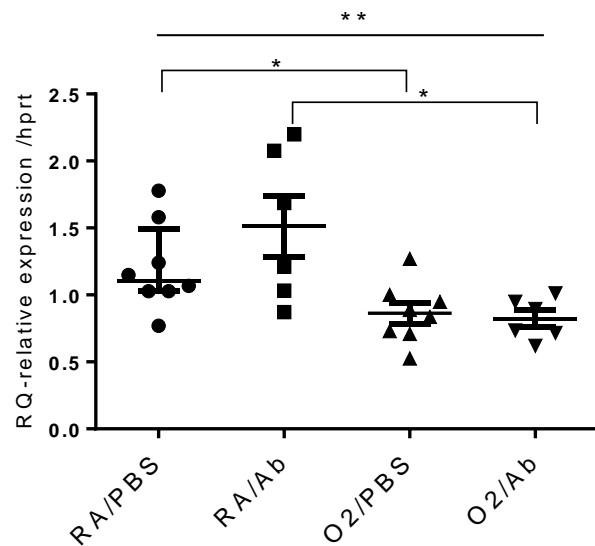


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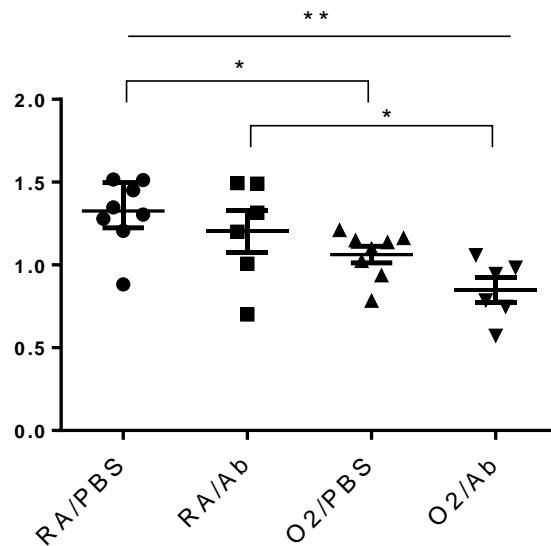




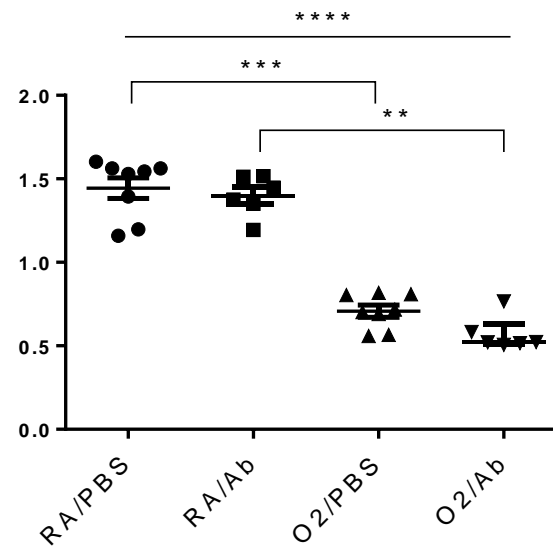
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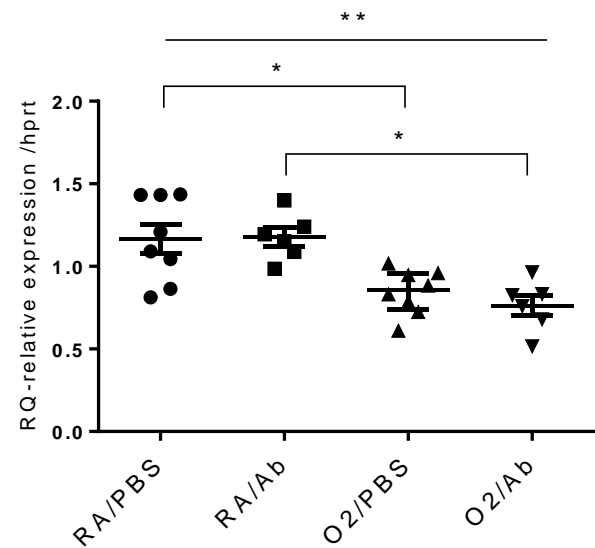
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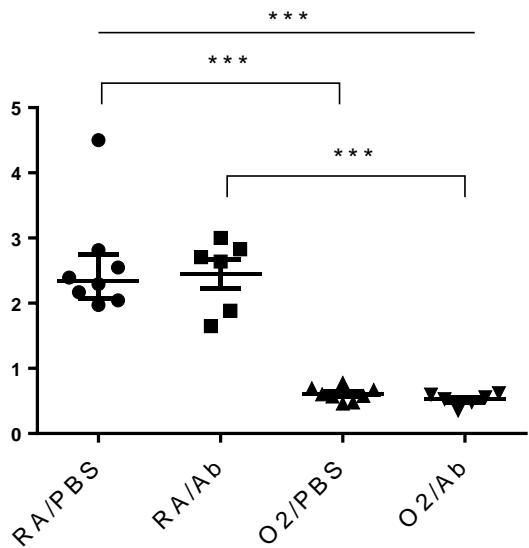
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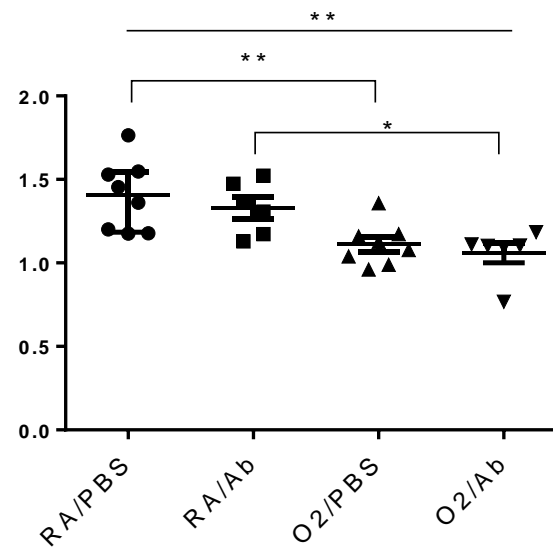
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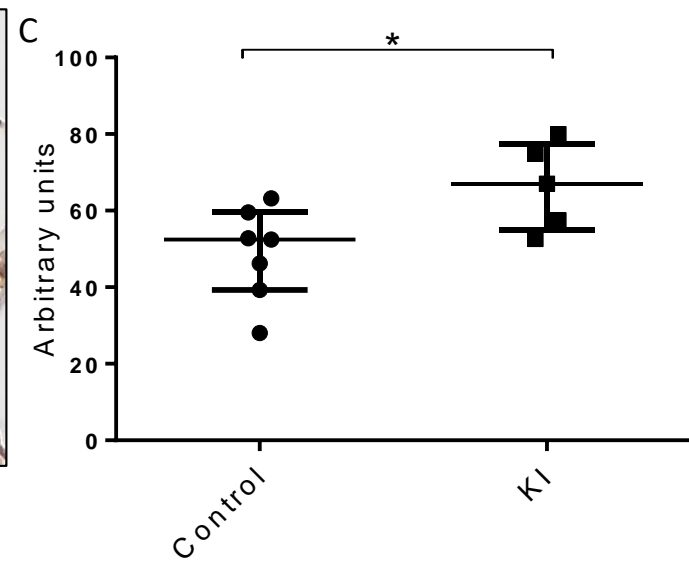
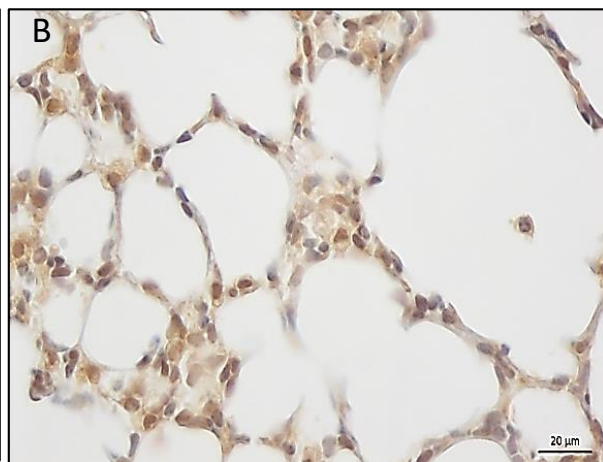
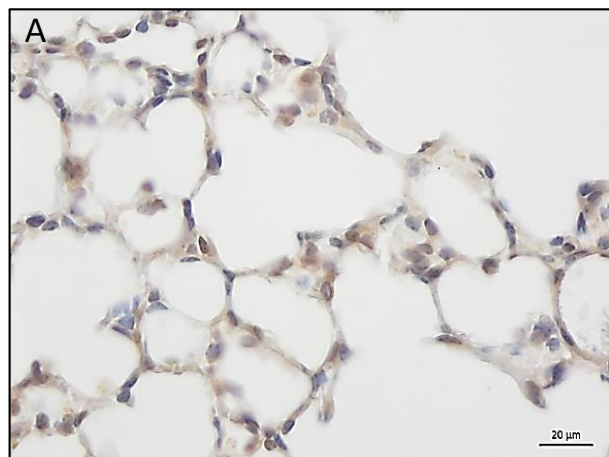


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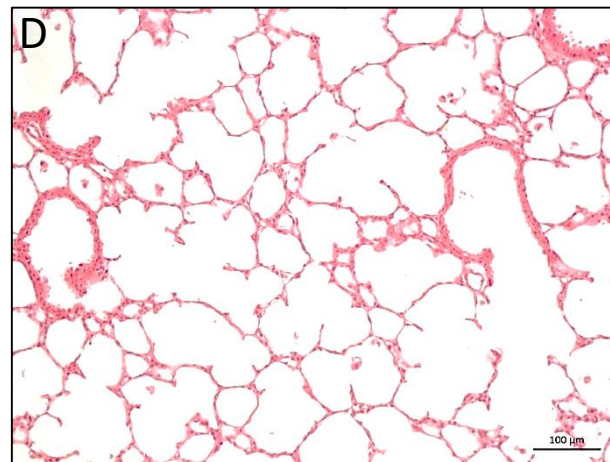
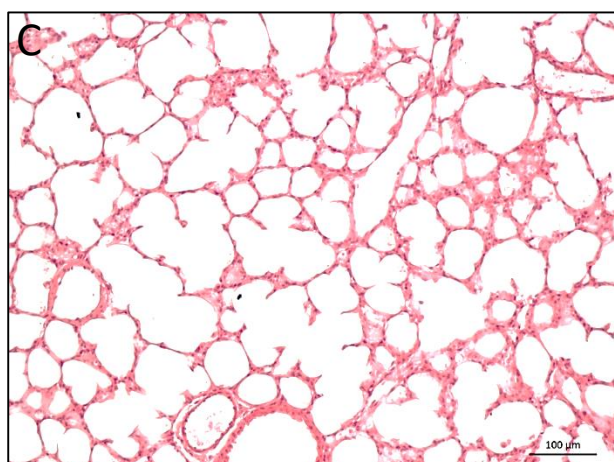
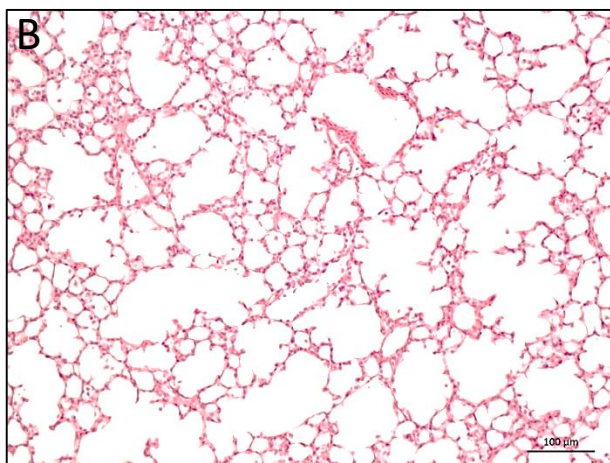
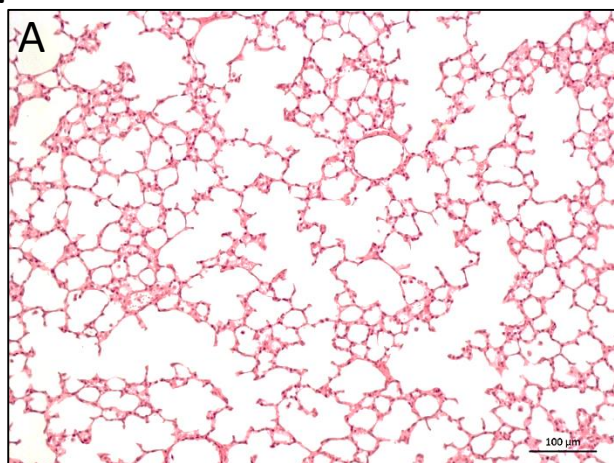


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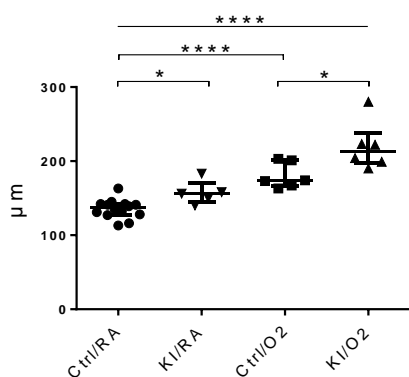




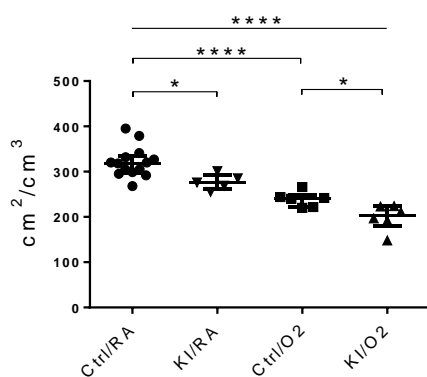
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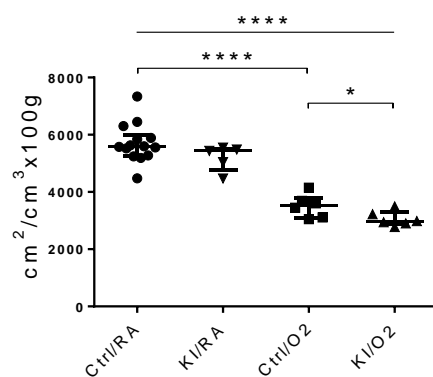
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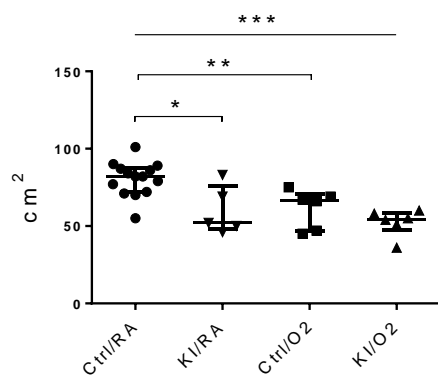
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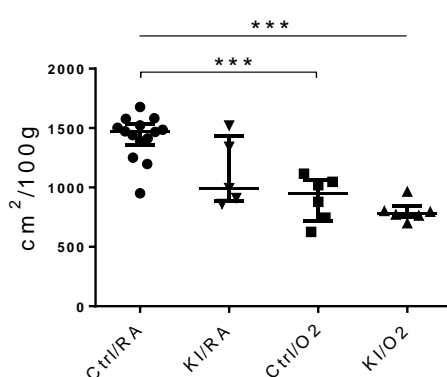
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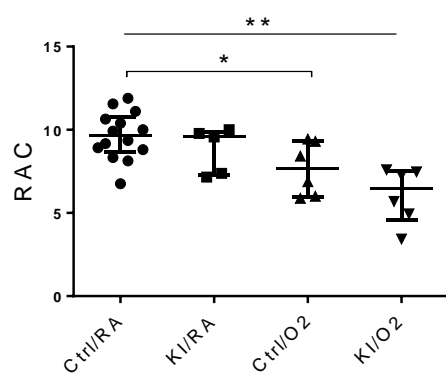
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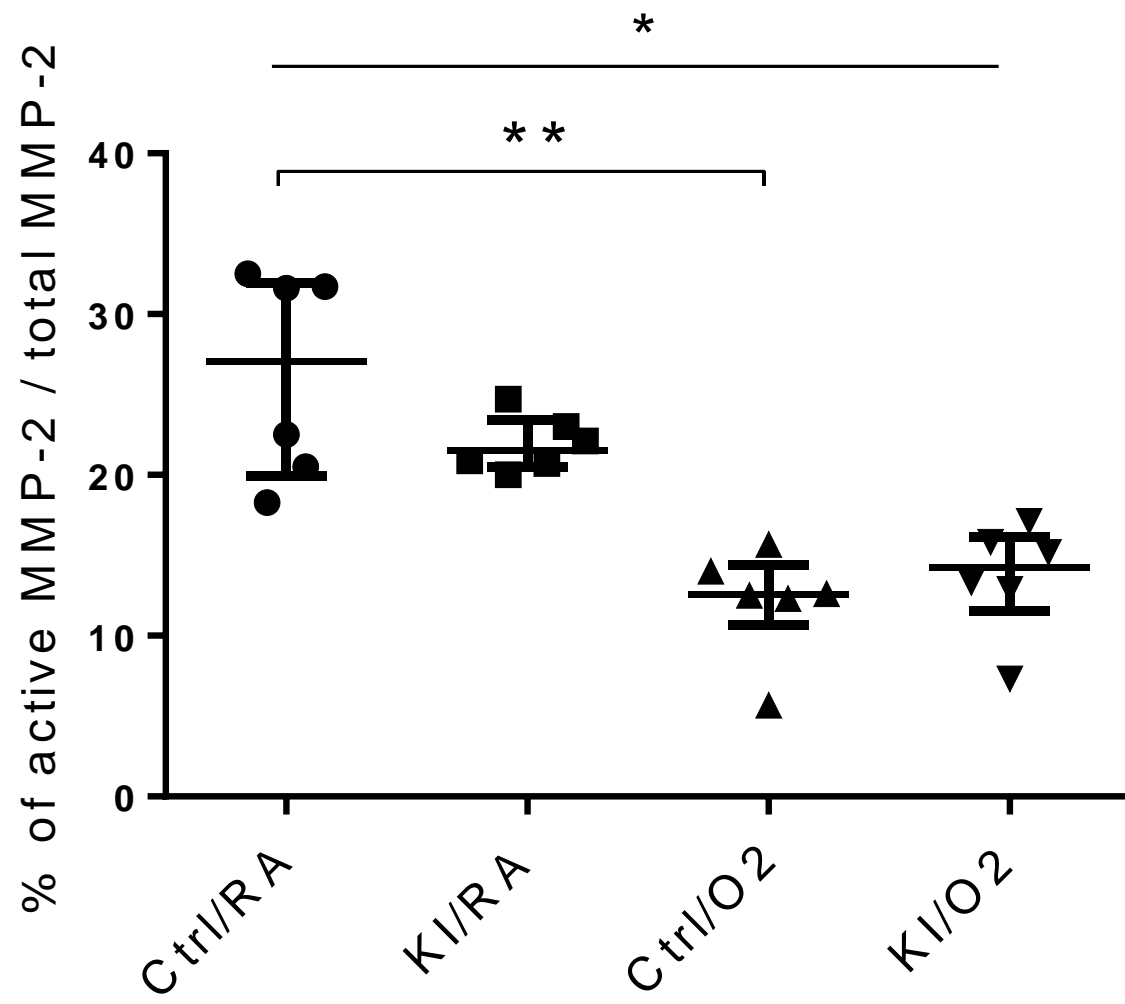


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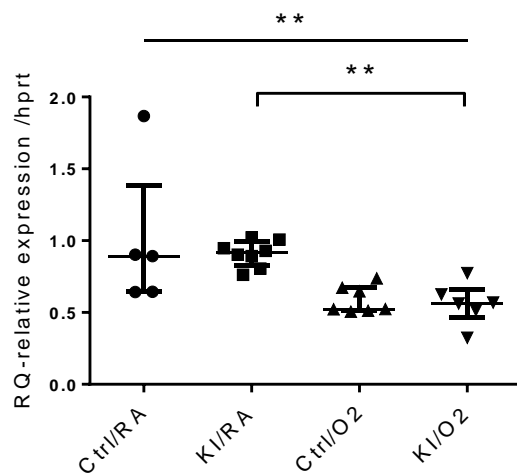


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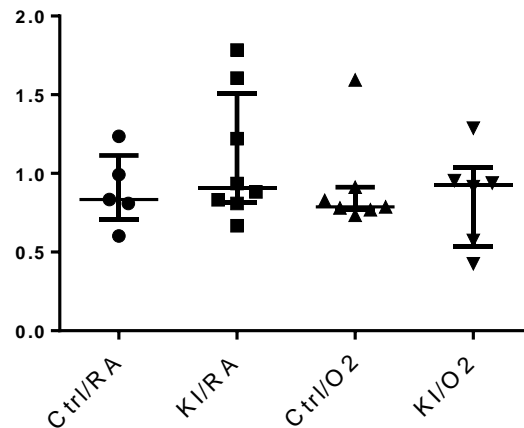




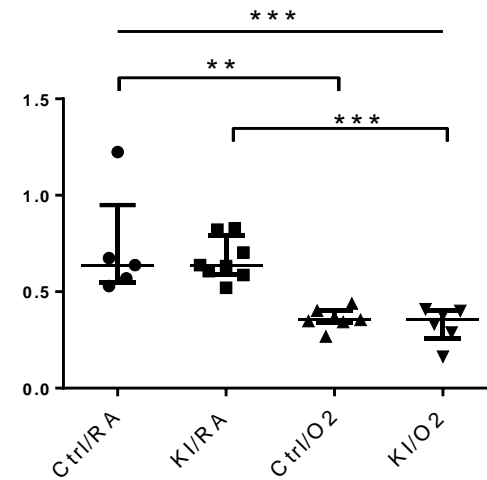
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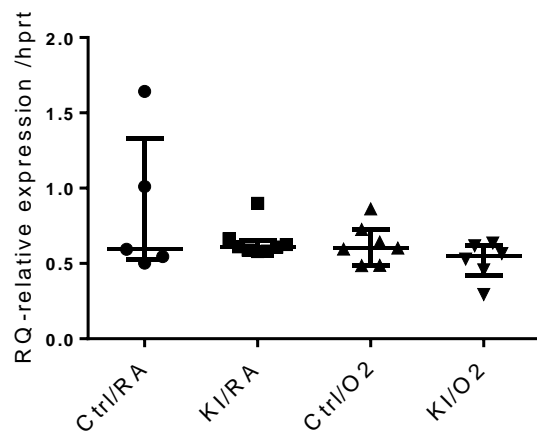
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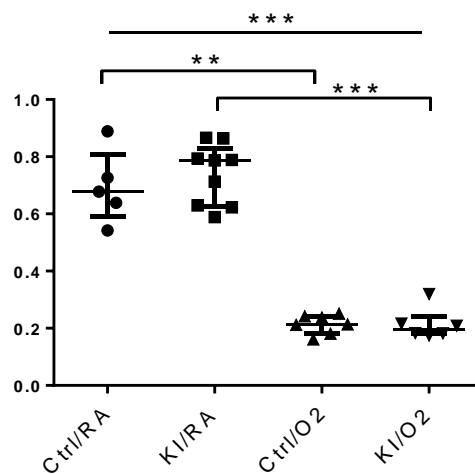
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