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Title: The link between regional tidal stretch and lung injury during mechanical ventilation

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Abstract

Aim: This study aimed to assess the association between regional tidal volume (V_T), regional functional residual capacity (FRC) and the expression of genes linked with ventilator-induced lung injury.

Methods: Two groups of BALB/c mice (n = 8 per group) were ventilated for 2 h using a protective or injurious ventilation strategy, with free-breathing mice as controls. Regional V_T and FRC of the ventilated mice was determined from analysis of high-resolution four-dimensional computed tomography (4D-CT) images, taken at baseline and after 2 h of ventilation and corrected for the volume of the region (i.e. specific [s]Vt and specific [s]FRC). RNA levels of 21 genes in ten different lung regions were quantified using qPCR array.

Results: sFRC at baseline varied regionally, independent of ventilation strategy, whereas sV_T varied regionally depending on ventilation strategy. The expression of *IL-6* ($P = 0.04$), *Ccl2* ($P < 0.01$) and *Ang 2* ($P < 0.05$) were associated with sV_T but not sFRC. The expression of seven other genes varied regionally (*IL-1β* and RAGE) or depended on ventilation strategy (*Nfe2l2*, *c-fos* and *Wnt1*) or both (*TNF*-α and *CxCl2*), but were not associated with regional sFRC or sV_T.

Conclusion: These observations suggest that regional inflammatory responses to mechanical ventilation are primarily driven by tidal stretch.

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Introduction

Acute respiratory distress syndrome (ARDS) has a high mortality rate (1-3). While the severity of ARDS is characterised by the degree of hypoxia (4), only a low percentage of ARDS patients died from hypoxemia; with the majority dying as a result of multisystem organ failure (MSOF) (5). Unfortunately, while mechanical ventilation is necessary for patients with ARDS, it may also directly contribute to MSOF by inducing inflammation (6, 7).

Mechanical ventilation in ARDS aims to provide adequate gas exchange while minimising lung injury, however, it may damage the lung in a process known as ventilator-induced lung injury (VILI) (8-10). The impact of high tidal volumes (over-distension) has been clearly demonstrated by the ARDSnet study, which showed 22% lower mortality in patients ventilated 6 mL/kg compared with those receiving a traditional tidal volume of 12 mL/kg (11). However, only one intervention has proved to be effective in reducing mortality in ARDS since this study; prone positioning for severe ARDS patients (1, 2, 12, 13). This highlights the importance of the lung mechanical response to ventilation in determining patient outcomes.

Identifying the optimum ventilation strategy to minimise VILI is complicated by the heterogeneous nature of ARDS (14). In addition, both over-distension and atelectrauma can occur within the same lung (15, 16) and can both can trigger an inflammatory response (biotrauma) (17-20). However, their relative contribution to overall biotrauma within an individual lung remains poorly understood (21). At the whole lung level, we have previously shown an association between over-distension and the presence of pulmonary oedema with protein leak and macrophage infiltration (21). However, we were unable to assess how these responses varied spatially, which may be an important determinant of outcome as studies have

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shown that ventilation causes a heterogeneous pattern of lung damage (15, 22) due to the inhomogeneous distribution of regional tidal strain (23).

The aim of this study was to assess the association between regional functional residual capacity (FRC) and regional tidal volume (V_T) and the expression of markers of lung injury in response to mechanical ventilation. We investigated this in a mouse model by combining a recently developed lung imaging technology, which allows image capture at high speed and high resolution over the entire breathing cycle (24), with gene expression analysis.

Methods

Animals

Six to nine week-old female BALB/c mice (Animal Research Platform, Monash University) were provided food and water *ad libitum* and housed in a 12:12 h light-dark cycle. All experiments were approved by the Monash University Animal Ethics Committees.

Animal preparation and ventilation

Mice were prepared as described previously (25). Briefly, they were anesthetised, trachestomised and mounted upright on a rotating stage (Zaber Technologies, Canada) in a customised holder. Mice were ventilated for 2 h with one of two protocols: 1) Protective (n = 16); 225 breaths/min, peak inspiratory pressure (PIP) 12 cmH_2O, PEEP 2 cmH_2O or, 2) Injurious (n = 16); 144 breaths/min, PIP 20 cmH_2O, PEEP 0 cmH_2O. Lung images of the ventilated groups were taken at baseline (H0) and after 2 h (H2) of ventilation. Mice were euthanased by overdose with sodium pentabarbitone (200 mg/kg) prior to processing of the lung tissue for gene expression (n = 8 per group) or immunohistochemistry (n = 8 per group). A separate group of mice (n = 8) served as unventilated controls for gene expression.

X-ray imaging

The x-ray imaging utilised a liquid metal-jet X-ray source (Excillum AB, Sweden), enabling high brightness and high-resolution imaging (26, 27). A high-speed detector (PaxScan, Varian Medical Systems, USA) was used to capture projection images at 30 frames per second for 4D-CT reconstructions (28).

Post-processing of imaging data

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We applied a 3D velocimetry technique to measure the regional tidal volume (V_T) (24, 28, 29), by analysing 400 frames per CT to calculate regional tissue expansion (24, 28, 29). In order to determine the regional FRC, we used the grayscale values (intensity) which were converted to Hounsfield Units (HU) to determine the fraction of air for each voxel.

To assess the regional V_T and FRC, the scans were segmented into ten regions (Fig 1). The airway tree was segmented (30) using a centreline tree extracted from an image of the airways filtered to accentuate cylindrical structures (31). The airway tree geometry was then used to segment the lung into five regions (left lobe and four right lobes; R1-R4), by assigning voxels to the nearest supplying airway (see (24, 29)). The left lobe data was further segmented into six (L1-L6) volumes (Fig 1).

Following segmentation of the lung imaging data, tissue expansion and volume of gas for each region was summed to provide the regional values of V_T and FRC, respectively. To correct for variation in regional lung volumes we calculated regional specific FRC (sFRC = regional FRC/regional lung volume), specific tidal volume (sV_T = regional V_T /regional lung volume), and regional lung distension (sFRC + sV_T). Global values for these indices were also calculated.

Regional gene expression

Following euthanasia, the lungs were removed *en bloc* and divided into 10 regions corresponding to the image segmentation (Fig 1). Each lung region was stored in RNA*later*® (Sigma, Australia) and RNA extraction was performed with a miRNeasy Mini Kit (Qiagen, Germany).

The expression of 21 genes was assessed using reverse transcription quantitative (q)PCR arrays according to the manufacturer's instructions (QIAGEN, Australia). VILI-related genes were selected with known roles in inflammation, surfactant production, epithelial and mesenchymal responses, transcription/cell signalling and coagulation (Table 1). qPCRs were performed on a LightCycler® 480 II instrument (Roche, Switzerland) in 96-well qPCR array plates. Gene expression relative to the house-keeping gene (*Rpl37*) was calculated using the $2^{-\Delta\Delta CT}$ method and expressed as fold change relative to the average gene expression of the L1 region in the free-breathing control group.

Immunohistochemistry

In a separate group of mice, following euthanasia, lungs were instillation-fixed *in situ* with 10% formalin at a trans-respiratory pressure of 10 cmH_2O for one hour. The trachea was ligated and the lung removed *en bloc* and submerged in formalin prior to transfer to 70% ethanol. The fixed lungs were embedded in paraffin and 5 μm coronal sections were taken at the midline. Immunohistochemistry was performed using a HRP/DAB (ABC) detection IHC Kit (Abcam, Australia). Antigen retrieval was performed in a pressure cooker using a solution of 1 mM EDTA in citrate buffer (pH 6.0) for 10 min. Sections were stained using anti-IL-6 (10 ng/μL, Ab208113, Abcam), anti-MCP-1 (5 ng/μL, Abcam, Australia), anti-p53 (0.5 ng/μL, Abcam, Australia) anti-neutrophil[NIMP-R14] (5 ng/μL, Abcam, Australia), or anti-Rabbit IgG polyclonal [Isotype Control] (5 ng/μL, Abcam, Australia) antibodies. The antigen-antibody reaction was visualised following the application of DAB substrate in DAB chromogen solution for 5 min and counterstaining with hematoxylin. Slides were dehydrated and clear mounted for light microscopy. Images of the enter section were captured and analysed using ImageJ. The regional levels of IL-6, Ccl2 (MCP-1) and p53 in the left lobe were estimated by calculating the proportion of the positive stain, per unit area of tissue, within each region after

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subtracting non-specific staining based on the intensity of isotype control stain in the adjacent section. The regional number of neutrophils was counted in randomly selected images, within each lung region, and quantified as the number of cells per unit area.

Data analysis

Differences in sFRC, sV_T, distension and relative gene expression, both between regions and between ventilation protocols, were assessed using two-way repeated measures ANOVA with Holm-Sidak *post hoc* tests (SigmaPlot v 12.5, Systat Software, USA). Between region protein expression data (immunohistochemistry) was assessed by one-way ANOVA with Holm-Sidak *post hoc* tests. Data were transformed where necessary to satisfy the assumptions of normality and homoscedasticity of the variances. Associations between regional sFRC, sV_T, distension and regional RNA levels were assessed using linear regression analysis.

Results

Lung motion

Qualitatively, lung stretch in the mice ventilated with the injurious protocol was heterogeneous and changed between baseline (H0) and after 2 hours (H2) of ventilation (Fig 2A, 2B), whereas lung stretch with the protective protocol was more homogeneous (Fig 2C, 2D).

sFRC, sV_T and distension at H0

At H0, global and regional sFRC were higher in the protective group than the injurious group ($P < 0.01$, Fig 3A). For both ventilation strategies, there were regional differences in sFRC ($P < 0.001$) where sFRC was lower in the more distal regions (L2, R2 and R4; $P < 0.05$). In general, regional sV_T was higher in the injurious group in most regions, with the exception of R4 (Fig 3B). In animals receiving injurious ventilation, regional sV_T was higher in proximal lung regions (L1 and R1; $P < 0.001$), whereas protective ventilation showed homogeneous regional sV_T at baseline (Fig 3B). Distension (sFRC + sV_T) followed a similar pattern to sV_T with higher distension in the injurious ventilation group; with the exception of the three lower regions (L5, $P = 0.51$; L6, $P = 0.54$ and R4, $P = 0.76$) (Fig 3C).

Change in sFRC, sV_T and regional distension after 2 hours of ventilation

After two hours of ventilation there was no change in sFRC (average change = 0.8%), sV_T ($\Delta = 3.2\%$) or regional distension ($\Delta = 1.1\%$) in the protective group (Fig 3D, 3E, 3F) compared to baseline. In contrast, sFRC reduced in all lung regions in the injurious group ($\Delta = 8.6\%$, Fig 3D), while the change in global sV_T was minimal ($\Delta = 1.2\%$, Fig 3E). On average, there was no significant change in global distension in the injurious group ($\Delta = 5.5\%$, $P = 0.76$, Fig 3F). There were, however, significant regional differences in sV_T and distension in the injurious group ($P < 0.001$, Fig 3E, 3F) between H0 and H2.

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Regional gene expression

The expression of *IL-6* (P = 0.02) and *Ccl2* (P < 0.001) varied regionally, depending on the ventilation strategy (Table 1). In contrast, the expression level of *Tnf-α* and C*xCl2* varied independently with both region and ventilation strategy (Table 1). The expression of *IL-1β, Ang-2,* and *RAGE* varied regionally, but there was no influence of ventilation strategy on the expression of these genes. Expression levels of three genes (*Wnt1*, P < 0.001; *c-fos*, P < 0.01; *Nfe2l2*, P < 0.001) varied depending on ventilation strategy, but there were no significant differences in levels between lung regions (Table 1).

There were no regional differences or associations with mechanical ventilation for the other genes measured (*Elane, MPO, SftpB, Cdh1, Ctnnb1, Egfr, TGFb1, Vim, Mapk1, Nfκb1,* and *Plat*) (Table 1).

The expression of *IL-6* in the protective group was higher than the free-breathing group while the expression in the injurious group was higher again. However, regional differences were limited to the injurious group with higher expression in the proximal (L1, L3 and R2) lung regions (Fig 4A). For *Ccl2*, the expression was elevated in some regions (R2, P = 0.03; L3, P < 0.01; L1, P = 0.03), but only in the injurious group.

Regional immunohistochemistry (IL-6, Ccl-2, p53 and neutrophils)

Ccl2 was expressed in the airway epithelial cells, inflammatory cells (primarily macrophages) and diffusely throughout the lung parenchyma (Fig 5A, B).The level of Ccl2 was highest in L1 and L3 (P < 0.05; Fig 6A) in the injurious group, which was consistent with the gene expression data (i.e. L1 and L3 had the highest *Ccl2* gene expression; Fig 4B). Interestingly, we saw the

same qualitative pattern in p53 protein expression (Fig 5B, C) whereby L1 and L3 had the highest expression ($P < 0.05$; Fig 6B). IL-6 was primarily expressed in the airway epithelium (*data not shown*). In contrast to the other proteins, there were no significant regional differences in IL-6 expression ($P = 0.89$; *data not shown*).

The number of neutrophils was higher ($P = 0.007$) in the protective group (17(2) per mm^2) than the injurious group (7(2) per mm^2), but there were no significant regional differences ($P = 0.40$; *data not shown*).

Association between regional gene expression and sFRC, sV_T, and regional distension

The only genes that were significantly associated with any of imaging measures were *IL-6, Ccl2 and Ang 2* (Table 2). Expression of *IL-6* was positively associated with sV_T and regional distension ($P < 0.05$) but not sFRC. Similarly, the expression of *Ccl2* was positively associated with sV_T and regional distension ($P < 0.01$) but not sFRC (Table 2 and Fig 7). Similarly, the expression of *Ang-2* was only, positively, associated with sV_T ($P < 0.05$) (Table 2 and Fig 7).

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Discussion

In this study, we assessed the impact of mechanical ventilation on regional lung volumes and gene expression. We found 1) a heterogeneous response to mechanical ventilation whereby sFRC varied regionally, independent of ventilation strategy, while sV_T and distension (sFRC + sV_T) varied regionally depending on the ventilation strategy used, 2) an overall reduction in sFRC in response to 2 h of injurious ventilation, 3) variations in regional gene expression levels that, in some cases (*IL-6, Ccl2*), depended on the ventilation strategy used, and 4) positive associations between the expression levels of *IL-6, Ccl2* and *Ang-2* and regional stretch but not sFRC. Collectively, these data highlight the complex and heterogeneous response of lung tissue to mechanical ventilation and how some, but not all, of the inflammatory response is linked to regional variations in tidal stretch. The lack of association between sFRC and altered gene expression suggests that overstretch is more detrimental than atelectasis.

We found that regional lung volumes varied in response to mechanical ventilation. This variability is consistent with that described in previous reports using larger animal models and static lung volume measures (22, 23). Of interest is the fact that the regional variation in sFRC we observed was consistent between the protective and injurious ventilation strategies; the more distal regions of the lung seemed to be more susceptible to under-ventilation (i.e. low sFRC) whereas they were protected from overstretch. There was minimal change in sFRC and sV_T over time in the protective group. In contrast, the injurious group showed a consistent loss of sFRC and significant variations in the sV_T response over time suggesting that this ventilation strategy alters the mechanical properties of the lung. The fact that we observed regional variations in lung stretch within minutes of mechanically ventilating the mice (i.e. at H0) and that these regional variations were associated with altered gene expression, suggests that local lung injury may develop very soon after the commencement of ventilation.

The regional variations we observed in sV_T were associated with the altered expression of *IL-6*, *Ccl2* and *Ang-2*. IL-6 is a key inflammatory cytokine (32) produced by epithelial cells and macrophages and is important in the progression of sepsis (17). Ccl2, or monocyte chemoattractant protein-1 (MCP-1), is responsible for the recruitment of macrophages to the site of inflammation (33, 34). The release of IL-6 and Ccl2 are closely linked (35), whereby IL-6 induces Ccl2 expression by peripheral blood mononuclear cells (36) while Ccl2 induces the release of IL-6 by human epithelial cells (37). Increasing levels of IL-6 has been demonstrated in response to injurious ventilation in clinical (20) and experimental (17) studies. Similarly, injurious mechanical ventilation is associated with increasing levels of Ccl2 in lung tissue (38), BAL and plasma samples (7, 38). Angiopoietin 2 is involved in the regulation of vascular endothelial permeability and is strongly linked to outcome in mechanically ventilated patients; particularly in ARDS (39). The link between the expression of this mediator and regional stretch highlights the role of over-stretch in contributing to ventilator induced vascular permeability and, potentially, edema; although we were not able to quantify the latter directly. While demonstration of upregulation of these genes in response to mechanical ventilation is not novel, the strong positive association between regional *IL-6, Ccl2* and *Ang 2* expression, regional sV_T and regional distension, and the absence of an association with sFRC, suggests that overstretch is the key driver of the expression of these mediators. Given the importance of these mediators in multi-organ dysfunction (7, 40) and patient mortality (39), our data suggest that avoidance of regional overstretch during initiation of ventilation may be a critical determinant of patient outcome.

While the expression levels of other genes (*CxCl2, TNF-α*, *c-fos, Nfe2l2* and *Wnt1*) were altered in response to the ventilation strategies used, their levels were not correlated with regional

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sFRC or sV_T. TNF-α and CxCl2 are pro-inflammatory cytokines that are elevated in response to mechanical ventilation (41). Nfe2l2, c-fos and Wnt1 are involved in transcription, cell signalling and mesenchymal responses respectively and have been previously associated with the response to mechanical ventilation (17, 42, 43). In the case of *TNF-α*, *c-fos* and *Wnt1*, the greatest expression was observed in the injurious group compared to the protective group, whereas the expression of *Nfe2l2* was equivalent between the protective and injurious groups and the expression of *CxCl2* was highest in the protective group compared to the injurious group. The expression pattern of *CxCl2* matched our neutrophil data whereby there were no regional differences in neutrophil numbers, but there was an association with ventilation such that the greatest neutrophilia was observed in the protective ventilation group. These observations are consistent with the notion that CxCl2 is involved in the recruitment of neutrophils during the early stages of inflammation (44). These variations suggest that the pattern of expression is correlated with the ventilation strategy. However, none of these genes were associated with regional sFRC or sV_T suggesting that local factors may not be driving the altered expression of these pathways. Alternatively, upregulation of the expression of these genes may be binary and based on a low threshold of activation in response to mechanical stretch, although this does not explain the expression pattern in *CxCl2*. Clearly, the link between regional overstretch and under ventilation, and the subsequent activation of biological pathways is complex. While we have identified a clear link between overstretch and the expression of *IL-6, Ccl2* and *Ang-2*, the mechanical and biological processes regulating the altered expression of the other genes warrants further investigation.

To gain further insight into the key processes, we quantified IL-6 and Ccl2 protein expression, to determine whether altered gene expression translated into protein synthesis, and p53, to gain further insight into the cellular response, by immunohistochemistry. We found that Ccl2

protein expression matched the gene data, while there was no difference in expression of IL-6 protein. It is unclear whether the latter was due a lack of translation or whether the timing was such that protein translation had not yet occurred. The pattern of p53 expression matched Ccl2. We assessed p53 as a potential marker of apoptosis (45), however, p53 has multiple functions in cell regulation. In this context, p53 has been shown to regulate Ccl2 expression (46); thus, the association between the expression of these proteins is perhaps not surprising. This suggests that the stretch induced production of Ccl2 may be regulated by p53, however, we are unable to rule out other roles for p53 in this context.

This study has several limitations. Firstly, our injurious ventilation strategy comprised of both high pressure and zero PEEP so we cannot separate the effect of each of these on the global expression levels of the genes we measured. However, our ability to calculate regional measures of sFRC and sV_T means that we were able to identify how these factors influence regional gene expression levels. We were also limited by the fact that our observations are primarily based on correlation between gene expression and sV_T; although the weight of evidence would suggest that the links we have made are causal and we were able to confirm increased *Ccl2* gene expression by immunohistochemistry.

In summary, we have demonstrated regional variations in sFRC and sV_T in response to mechanical ventilation. By measuring the expression levels of a suite of genes, we were also able to assess the link between the mechanical response of the lung and alterations in regional gene expression. Interestingly, no alterations in gene expression levels were associated with markers of atelectasis whereas alterations in expression of *IL-6* and *Ccl2* were clearly linked to regional overstretch. There were a number of genes with altered expression levels with particular ventilation strategies, but not the regional mechanical response, which warrants

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further investigation. In the context of therapeutic approaches to improve outcomes in critically ill patients who are mechanically ventilated, our observations suggest that pro-inflammatory pathways are activated very early in response to mechanical stretch. What is unclear is whether this response resolves when stretch is subsequently reduced. Our data also indicate that IL-6, Ccl2 and Ang-2 are the most sensitive to regional variation in stretch. However, the relative pathological significance of these mediators is unclear with studies suggesting that up-regulation of IL-6 may be protective (47) while up-regulation of Ang-2 is detrimental (48); thus the net-effect may depend on the relative expression of these pathways. Further exploration of this interaction is necessary to further understand the pathogenesis of ventilator induced lung injury. Our study clearly highlights the complexity of the link between mechanical ventilation and biotrauma.

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Figure legends

Figure 1. Regional lung segmentation. Schematic showing the segmentation approach for the assessment of regional lung volumes. This segmentation corresponded to the tissue sampling process for the assessment of gene expression.

Figure 2. Imaging of lung motion during ventilation. Representative transverse images from the apex (top) to the base (bottom) of a mouse ventilated with an injurious strategy at baseline (A), and after 2 hours of ventilation (B), and a mouse ventilated with protective strategy at baseline (C) and after 2 hours ventilation (D). Tidal volume contours (arbitrary units) were normalised to the size of the whole lung.

Figure 3. Effect of different ventilation strategies on lung volumes. Box plots (median, interquartile range, and 10th to 90th percentile) for sFRC, sV_T and sFRC + sV_T at baseline (panels A, B, C, respectively) and the proportional change in sFRC, sV_T and sFRC + sV_T (panels D,E, F, respectively) after 2 hours of ventilation relative to baseline for each of ten lung regions in mice ventilated with a protective or injurious strategy. * and ** indicate $P < 0.05$ and $P < 0.001$ respectively between ventilated strategies. † and †† indicates $P < 0.05$ and $P < 0.001$, respectively, compared with same region in the protective group. N = 8 mice per group.

Figure 4. Regional gene expression measured by qPCR array. Relative fold change of RNA levels was calculated using $2^{-\Delta\Delta CT}$ method relative to the housekeeper (*Rlp37*) and average C_T of the L1 region in the free-breathing group for *IL-6* (A), *Ccl2* (B), *TNF-α* (C) and *IL-1β* (D). * indicates $p < 0.05$. Data are shown as mean (SD). N = 8 per group.

Figure 5. Qualitative regional expression of Ccl2 and p53. Ccl2 staining (A, B) was localised to the airway epithelium, inflammatory cells (macrophages; arrow) and was also expressed diffusely throughout the parenchyma. The expression pattern of p53 (C, D) was similar to Ccl2. There was regional variation (quantified in Figure 6) such that, for example, the expression levels of Ccl2 and p53 were higher in the L1 (A, C) region than the L2 (B, D) region.

Figure 6. Regional expression of Ccl2 and p53 protein in the left lobe of the injurious ventilation group. The regional (L1-L6) proportion of positive staining tissue for Ccl2 (A) and p53 (B) protein (based on immunohistochemistry) per unit tissue area after subtracting the background level of stain from the isotype control. * indicates $p < 0.05$. Data are shown as mean(SD). N = 6 per group.

Figure 7. Relationship between regional gene expression (*IL-6*, *Ccl-2* and *Ang-2*) and lung volumes. Scatterplots showing the relationship between the fold change in regional gene expression of *IL-6* (A, B), *Ccl2* (C, D) and *Ang-2* (E, F) and regional specific FRC (sFRC; A, C, E) and regional specific tidal volume (sV_T, B, D, F) in the injurious group. Lines represent the predicted association based on linear regression analysis.

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Table 1: Relationship between gene expression, ventilation strategy and lung region. P-values for the main effects (ventilation, region) and the interaction term (ventilation x region) based on a two-way ANOVA for RNA expression relative to the house keeping gene (*Rlp37*).

Gene function	Gene name	Ventilation	Region	Ventilation x Region
Inflammation	*Ccl2*	0.07	**<0.001**	**<0.001**
	CxCl2	**<0.001**	**0.04**	0.48
	Elane	0.12	0.30	0.13
	IL-1β	0.48	**0.02**	0.72
	IL-6	**<0.001**	0.08	**0.02**
	Mpo	0.14	0.35	0.70
	Tnf-α	**<0.01**	**<0.01**	0.86
	RAGE	0.34	**0.01**	0.86
	Ang-2	0.73	**<0.001**	0.75
Surfactant	*Sftpb*	0.67	0.95	0.81
Epithelial-mesenchymal response	*Cdh1*	0.81	0.33	0.88
	Ctnnb1	0.65	0.87	0.78
	Egfr	0.22	0.24	0.88
	Tgfb1	0.39	0.67	0.90
	Vim	0.88	0.08	0.78
	Wnt1	**<0.001**	0.16	0.29
Transcription, cell signalling	*c-fos*	**<0.01**	0.59	0.93
	Mapk1	0.99	0.37	0.54
	Nfe2l2	**<0.001**	0.81	0.87
	Nfkb1	0.78	0.51	0.94
Anticoagulant	*Plat*	0.14	0.14	0.97

Table 2: Univariate relationships between gene expression and regional lung volume indices. R^2 and P values from the linear regression analysis examining the association between gene expression and regional specific functional residual capacity (sFRC), specific tidal volume (sV_T) and distension (sFRC + sV_T). Only genes that showed significant variations by region and/or in response to ventilation were included in this analysis.

Gene	sFRC		sV_T		Distension (sFRC + sV_T)	
	R^2	*P-value*	R^2	*P-value*	R^2	*P-value*
Ccl2	0.002	0.76	**0.157**	**<0.01**	**0.121**	**<0.01**
CxCl2	<0.001	0.92	0.038	0.14	0.033	0.17
IL-1β	0.016	0.34	0.048	0.09	0.058	0.06
IL-6	0.009	0.47	**0.063**	**0.05**	**0.068**	**0.04**
c-fos	<0.001	0.92	0.025	0.23	0.022	0.26
Tnf-α	0.015	0.35	0.032	0.17	0.042	0.12
Wnt1	0.023	0.25	0.007	0.54	0.016	0.34
Nfe2l2	0.036	0.15	<0.001	0.98	0.04	0.64
RAGE	0.005	0.58	0.018	0.31	0.009	0.46
Ang-2	0.01	0.45	**0.063**	**0.05**	0.038	0.14

sFRC = specific functional residual capacity

sV_T = specific tidal volume

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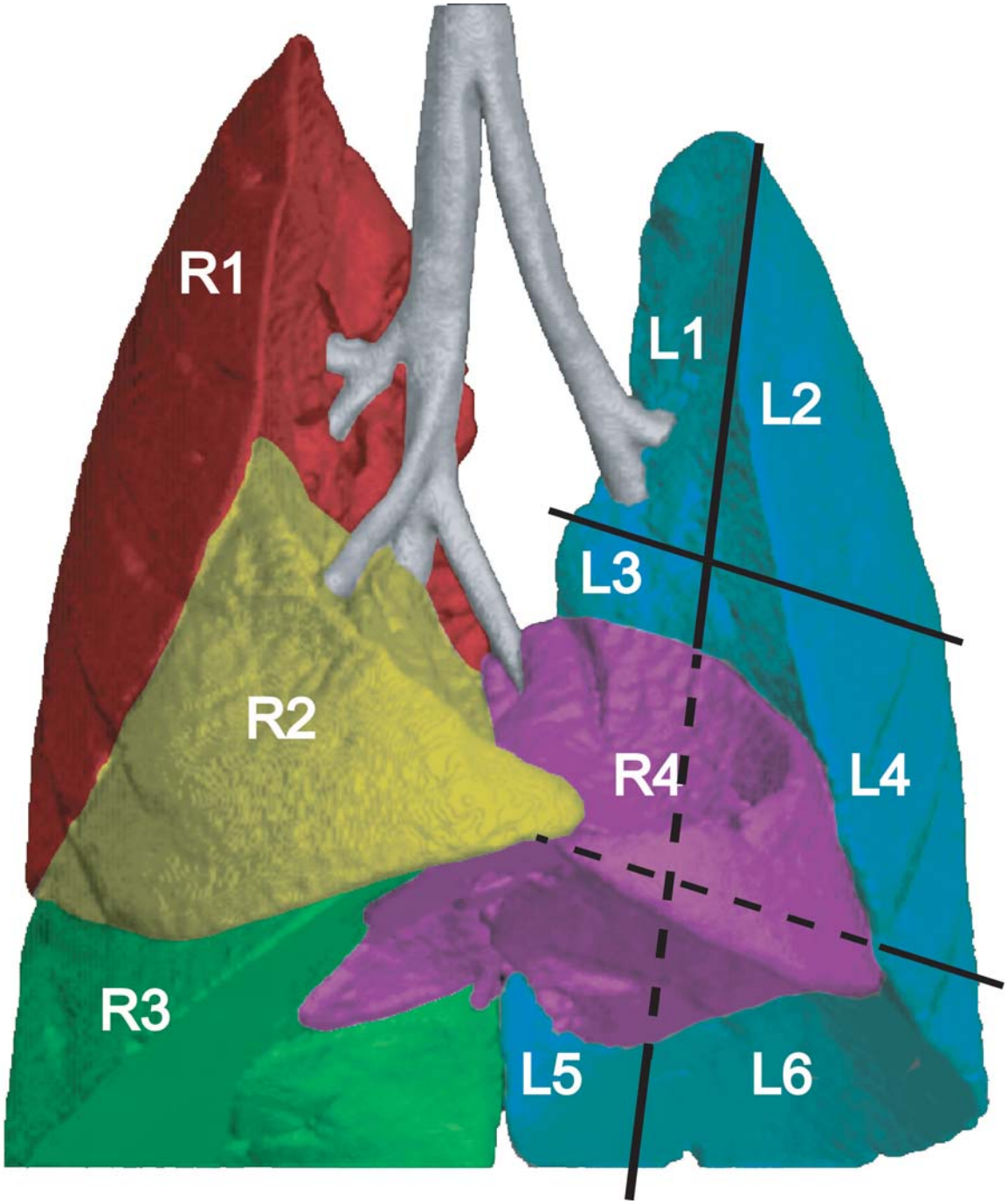

Figure 1

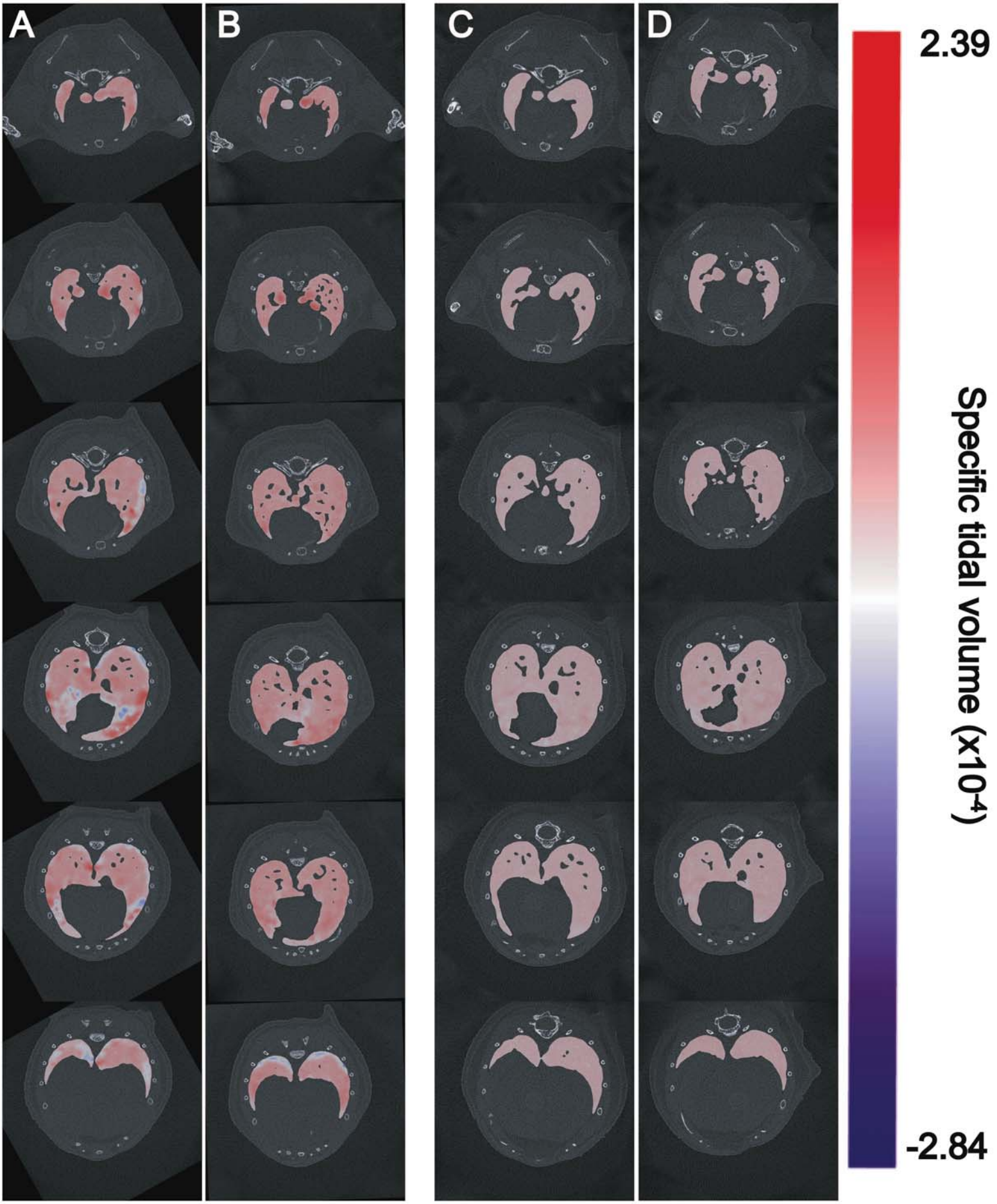

Figure 2

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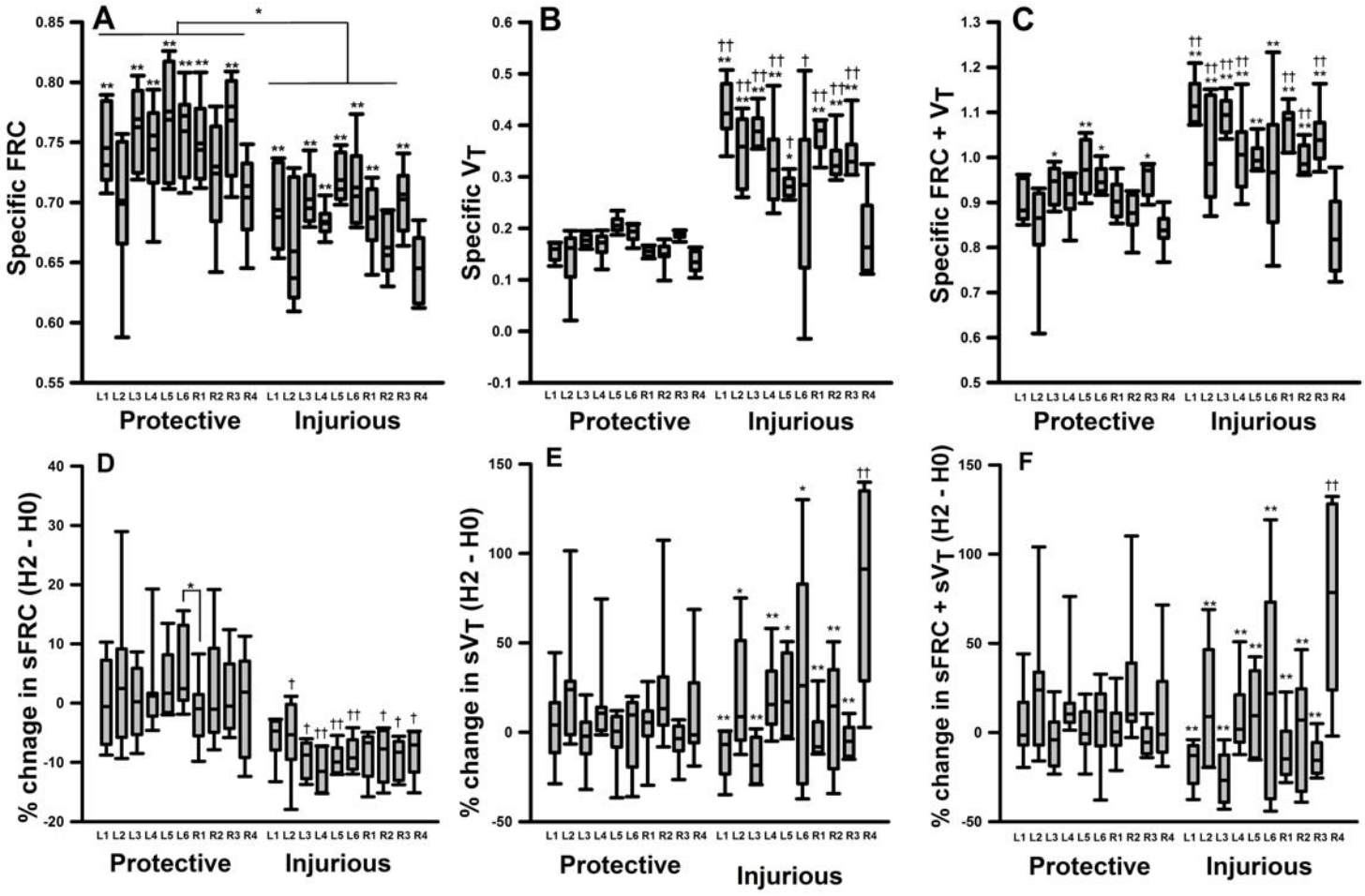

Figure 3

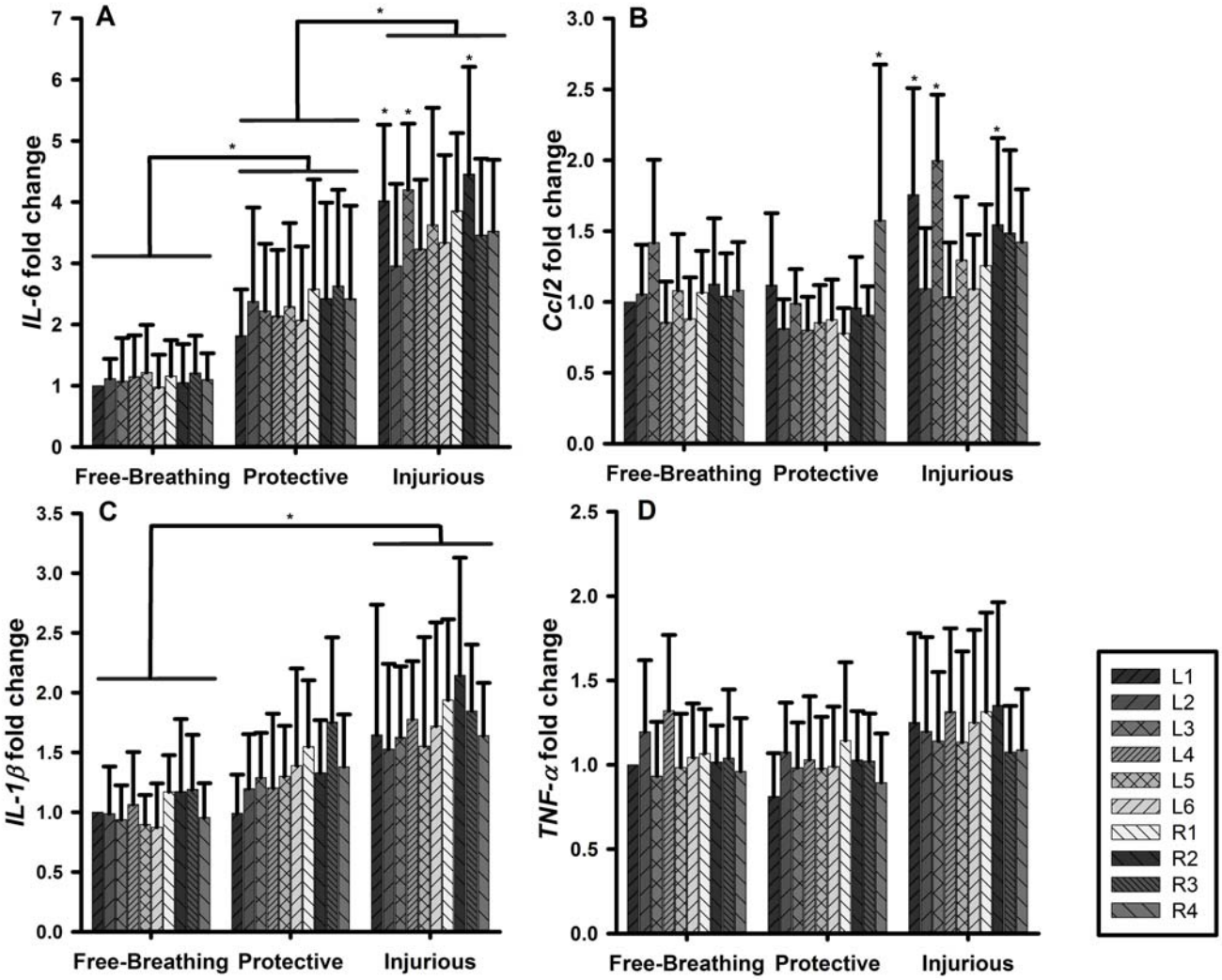

Figure 4

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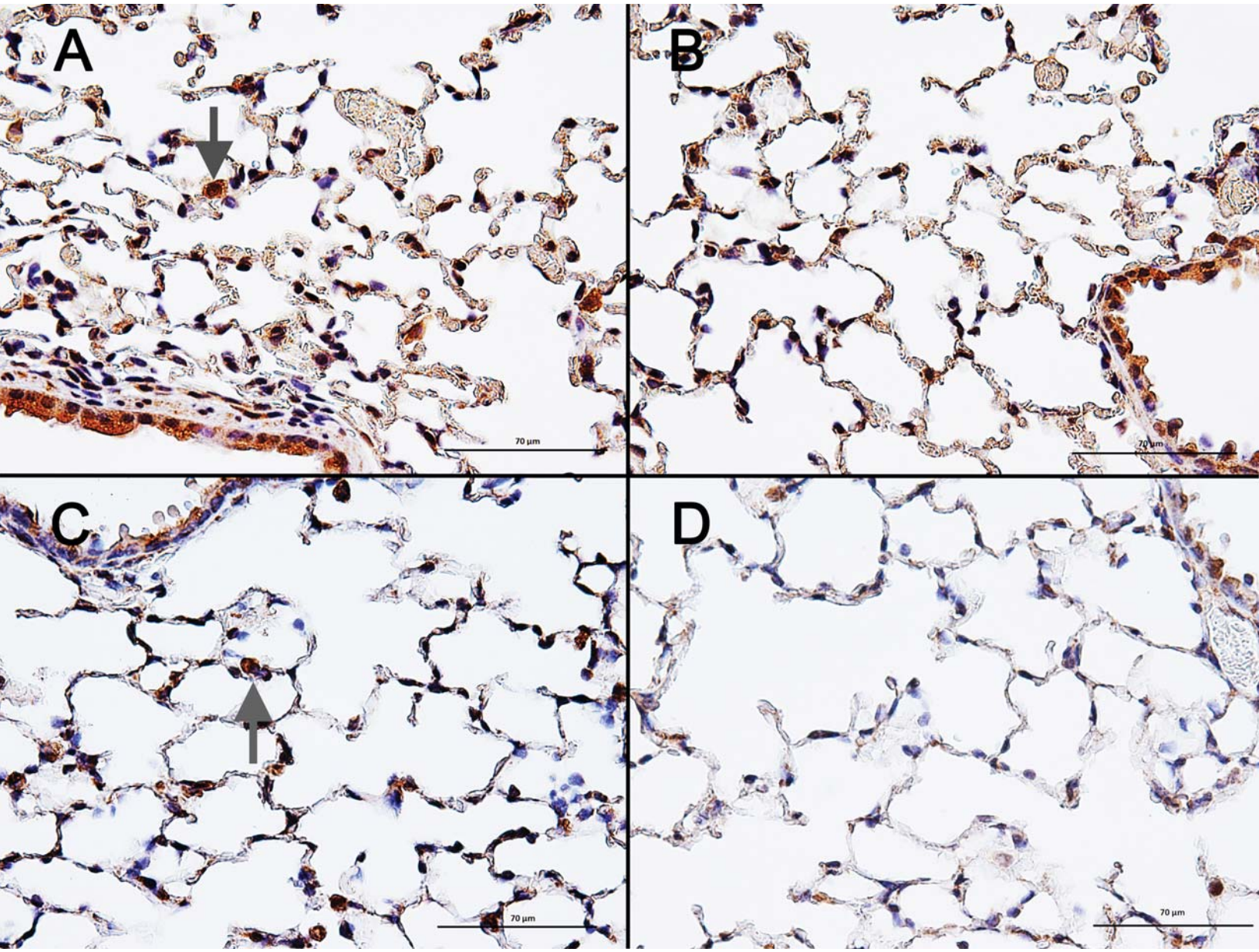

Figure 5

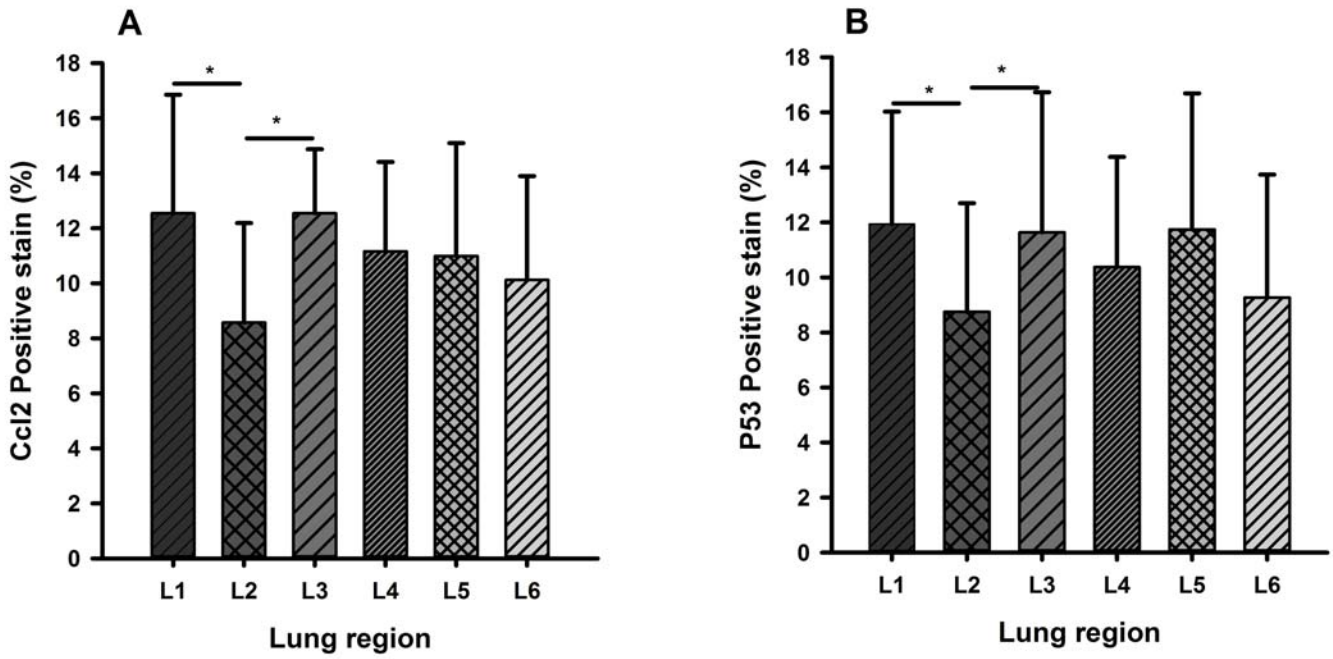

Figure 6

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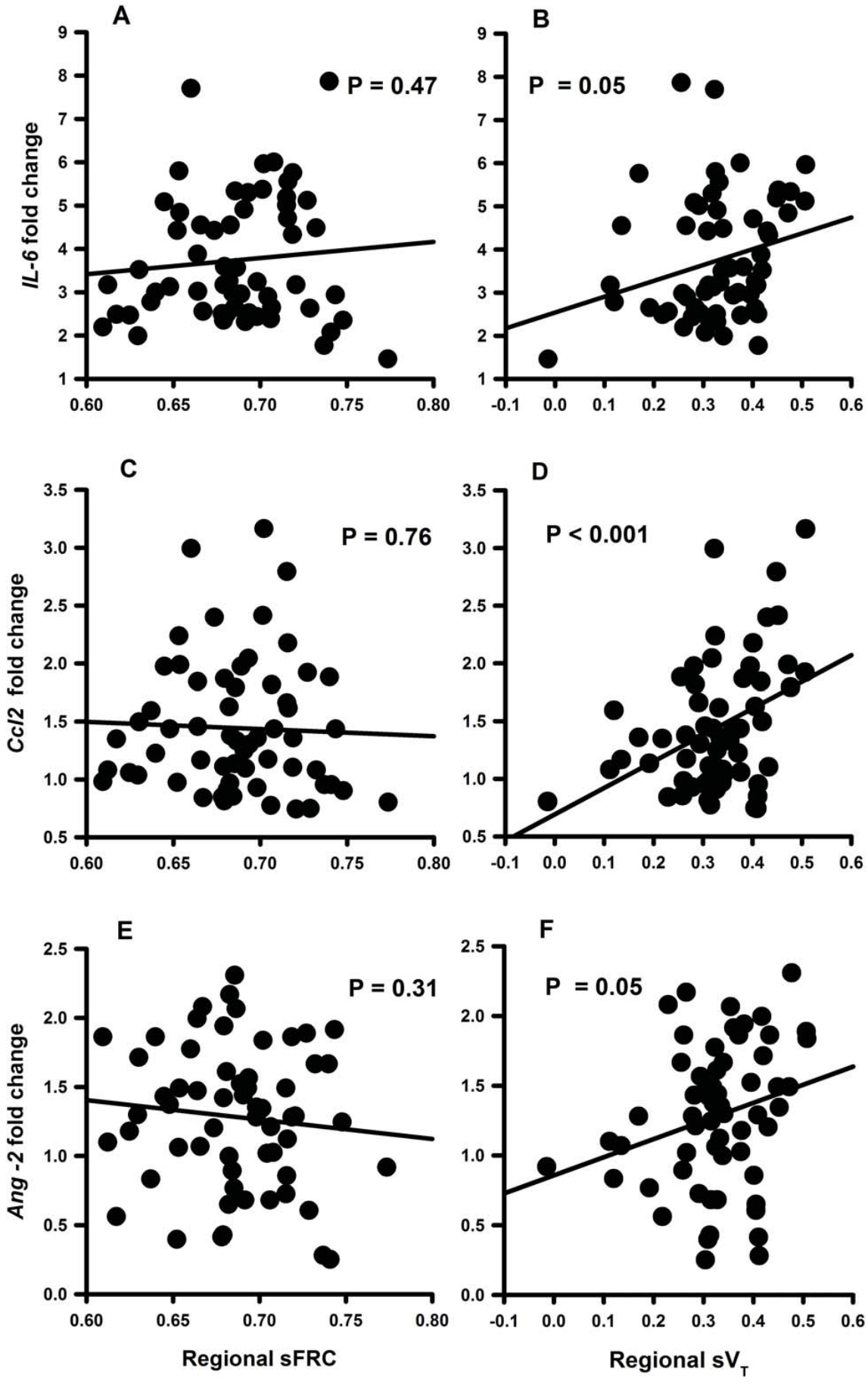

Figure 7