

Pro-inflammatory cytokine expression and acute neuronal injury in the nTS following bleomycin-induced lung-injury

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Introduction:

Acute respiratory distress syndrome (ARDS) afflicts 200,000 individuals in the U.S. annually. During the acute phase of ARDS, inflammation in the lung is triggered by local or systemic infection. Localized lung inflammation can initiate inflammatory responses in the CNS, often affecting areas of the brainstem crucial for respiratory control. Previously we showed an increase in caudal brainstem IL-1beta expression within neurons of the *nucleus tractus solitarius* (nTS) using bleomycin (bleo), a chemotherapeutic agent known to induce lung injury in rats. We hypothesized that, at 7 days—the peak of the bleo-induced lung inflammatory response—elevated pro-inflammatory cytokine expression and IL-1R signaling in the nTS are associated with acute neuronal injury. We used immunohistochemistry to quantify expression of several pro-inflammatory cytokines, IL-1R signaling factors, and heat shock protein 70/72 (HSP-70) at 7d post bleo injury within the caudal nTS. To test this hypothesis, Sprague-Dawley rats (>P21) were anesthetized and instilled with bleo or PBS. At 7 days post-injury, rats were transcardially perfused/fixed for immunohistochemical analysis. We measured increased immunoreactivity for IL-1beta, IL-6, TNF-alpha, COX-2, mPGES-1 in the caudal nTS. We observed no changes in microglial activity (measured by Iba1) but HSP-72, a marker of neuronal injury, was increased at 7d post bleo. These data suggest that localized lung inflammation promotes pro-inflammatory cytokine increases in a brainstem area critical for sensory integration that may lead to neuronal injury.

Methods

- Intra-tracheal instillation of bleomycin (3.0U) or saline was performed on adult male Sprague-Dawley rats (N=14). At 7d animals were perfused transcardially with phosphate buffer saline (PBS) and fixed with paraformaldehyde (PFA) 4%.
- Brainstems were removed and post-fixed in PFA 4%, cryoprotected in 30% sucrose, embedded in a tissue freezing medium, sectioned (20 μ m), placed serially on quintuplicate slides and stored at -20°C until immunostaining.
- Slides were stained immunohistochemically (IHC) for Iba1, IL-1 β , TNF- α , IL-6, COX-2, mPGES-1 or hsp70/72. Dual fluorescent IHC was used to examine the cellular subtypes involved in mediating the inflammatory response within the nTS.
- Sections were photographed in the area of interest at the same light intensity and exposure time; white balance was performed using imaging software. Staining intensity was assessed in anatomically equivalent sections. Unbiased stereology was performed on anatomically equivalent sections (Stereologer System 2001, Version 2).

Results:

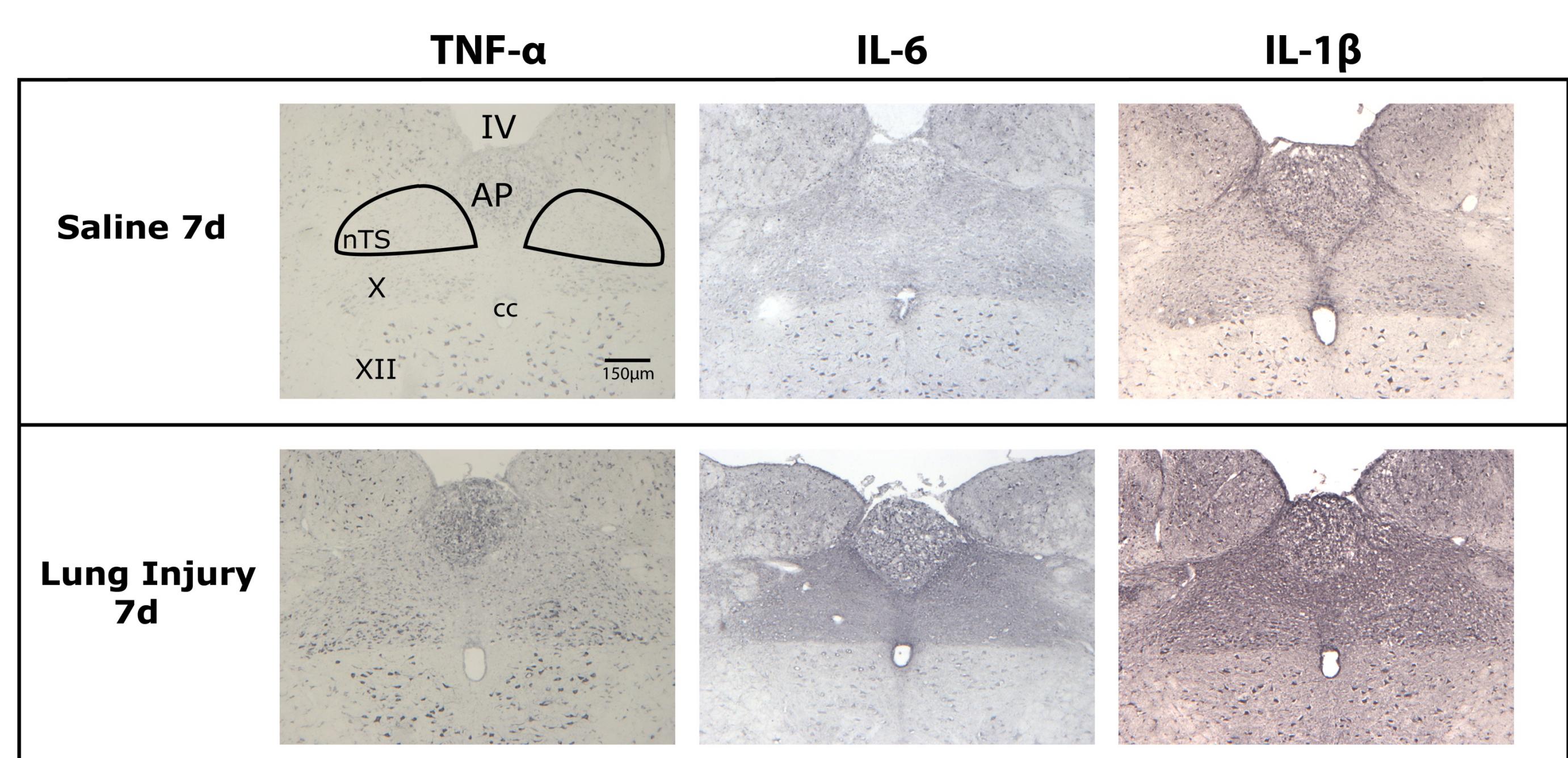


Figure 1: IHC staining reveals increased expression of IL-1 β , TNF- α and IL-6 at 7d post lung injury. Enhanced pro-inflammatory cytokine immunoreactivity was observed most prominently throughout the caudal nTS, area postrema and dorsal motor nucleus of the vagus (X). The hypoglossal nucleus (XII) did not show enhanced cytokine expression, suggesting this response to bleo-induced lung injury is isolated to the dorsal vagal complex. Immunoreactivity was observed in cells sharing the morphology of astrocytes, microglia and neurons.

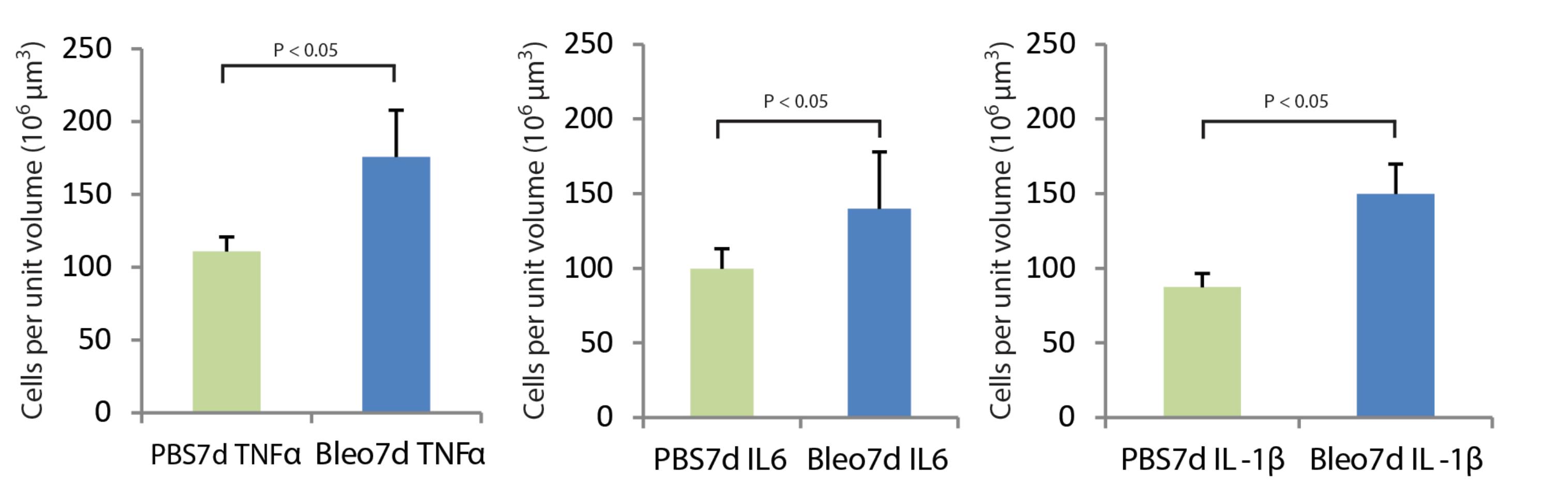


Figure 2: Unbiased stereological quantification for IL-1 β , TNF- α and IL-6 immunoreactivity shows significantly elevated pro-inflammatory cytokine levels in the nTS at 7d post lung injury. An average volume of $250 \times 10^6 \mu\text{m}^3$ was interrogated, consisting of 20 μ m sections from bregma -16.68mm to -14.08. Cytokine levels are expressed as immunoreactive cells per $10^6 \mu\text{m}^3$ of tissue (N=10).

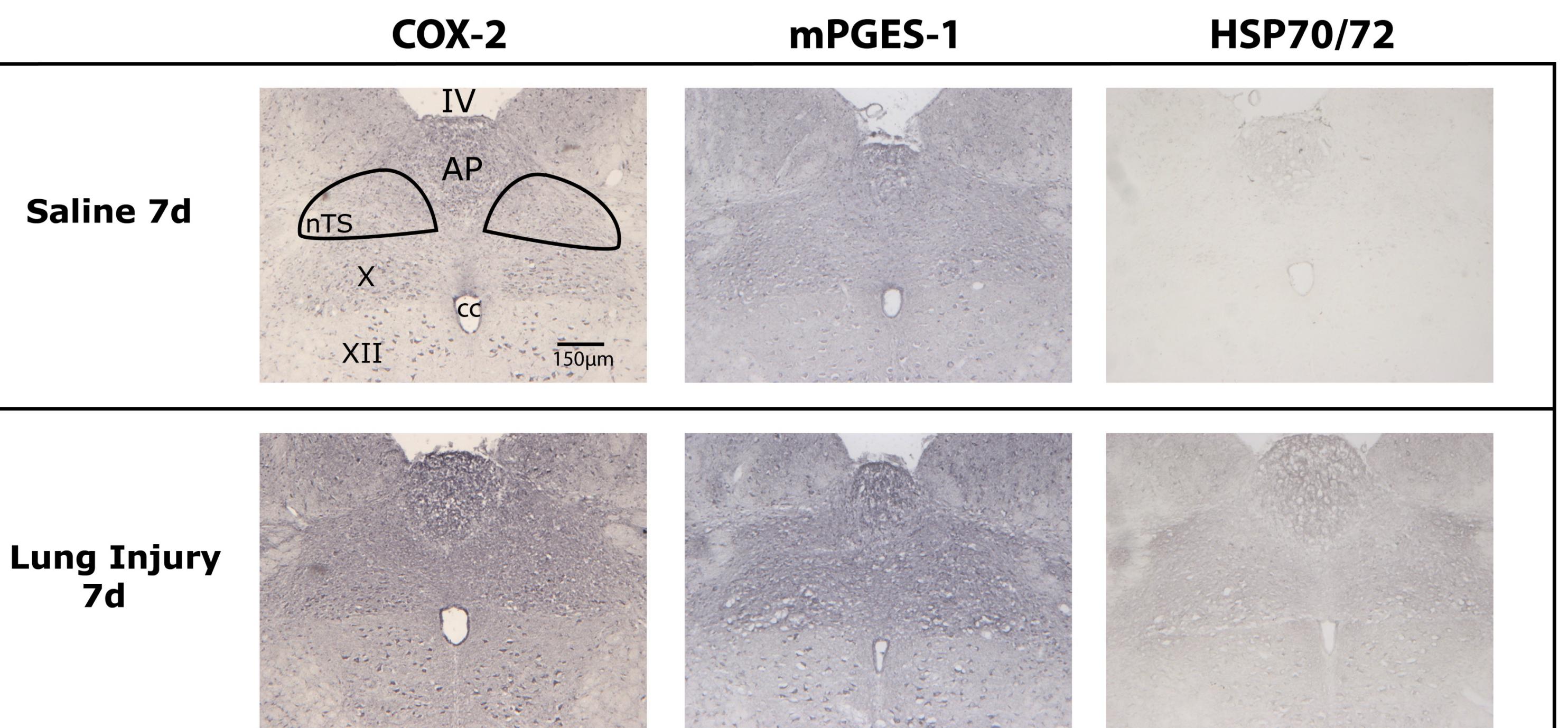


Figure 3: IHC staining shows enhanced expression for downstream signaling targets of IL-1R, and evidence of cell injury in the nTS at 7d post lung injury. Cyclooxygenase 2 (COX-2) and membrane associated prostaglandin E synthase 1 (mPGES-1) immunoreactivity was elevated throughout the dorsal vagal complex but did not appear enhanced in the hypoglossal nucleus. These findings are consistent with the expression pattern we observed for IL-1 β following lung injury. Heat shock proteins are upregulated during injury within the brain and are a component of the neuroprotective response to neuronal injury. IHC staining revealed expression of the molecular chaperone and injury marker HSP-70/72 throughout the dorsal vagal complex following bleomycin induced lung injury. Increased immunoreactivity was observed in the DMN, nTS and area postrema. The hypoglossal showed enhanced immunoreactivity for HSP-70/72. This response was minor in control animals. N=10

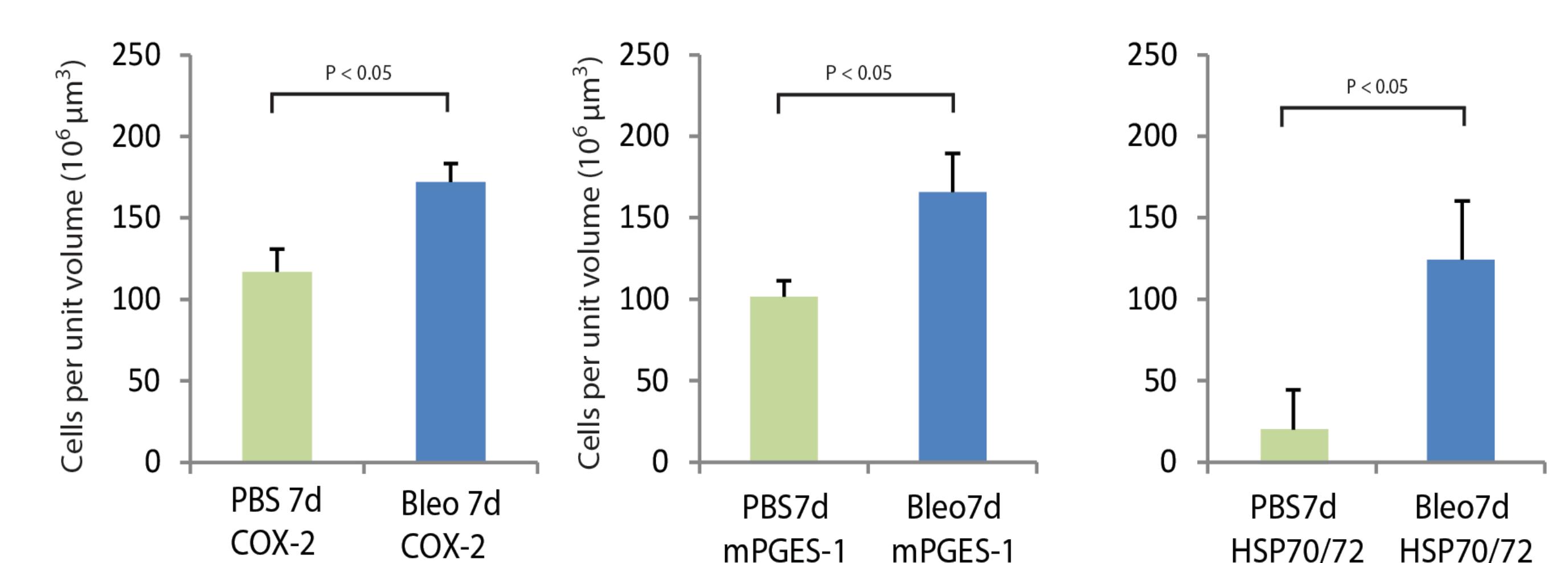
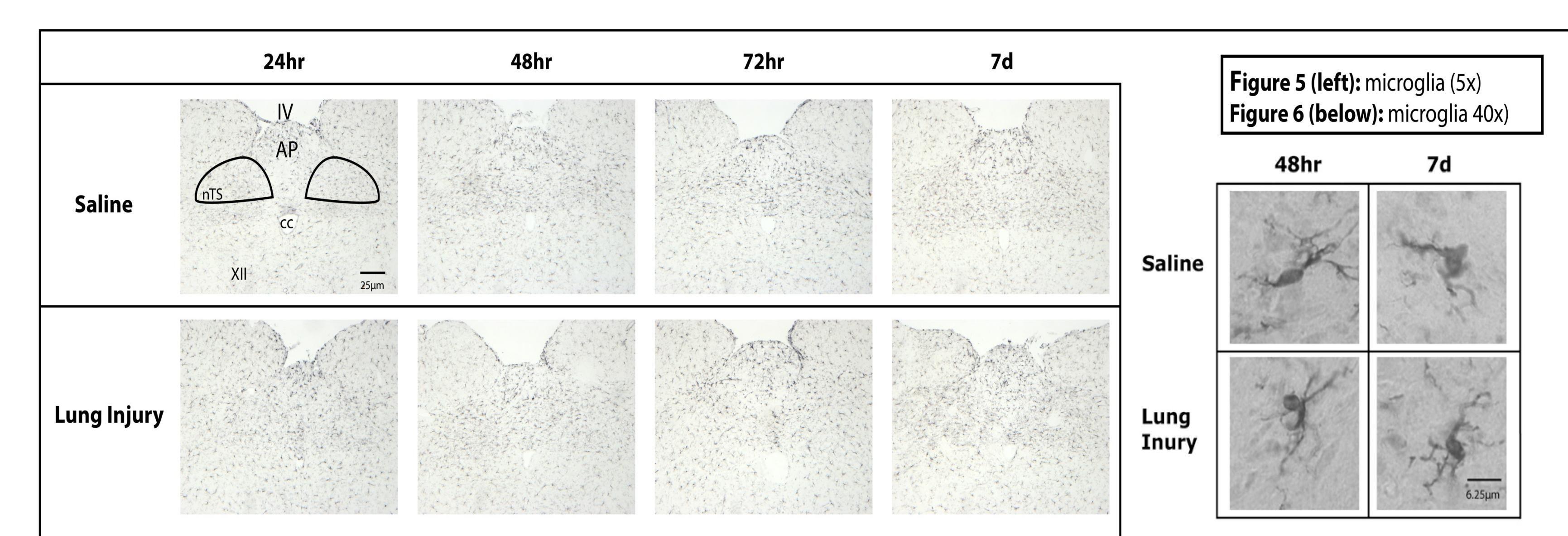


Figure 4: Unbiased stereological quantification for COX-2, mPGES-1 and HSP-70/72 immunoreactivity in the caudal nTS shows a statistically significant increase at 7d post lung injury. We hypothesized that the presence of elevated proinflammatory cytokines as well as an enhanced PGE₂ synthesis pathway may be associated with damage to the dorsal vagal complex. Stereological quantification of HSP-70/72 showed a dramatic increase in expression at 7d post lung injury (N=10).



Figures 5 & 6: Microglial activity appears unaltered following Bleomycin-induced lung injury. Microglia have been reported to release proinflammatory cytokines following various brain insults. We speculated that the increase in IL-1 β , TNF- α and IL-6 in the nTS following lung injury was associated with increased microglial activation. Immunohistochemical staining for Iba1 (ionized calcium binding adaptor molecule 1) did not reveal observable changes in the density or morphology of microglia in the nTS at 7d. We further speculated that microglial activation may precede cytokine release and so we examined Iba1 immunoreactivity at 24hr, 48hr, and 72hr post bleomycin-induced injury. We did not observe changes in microglial activation profiles at any of these times (N=12)

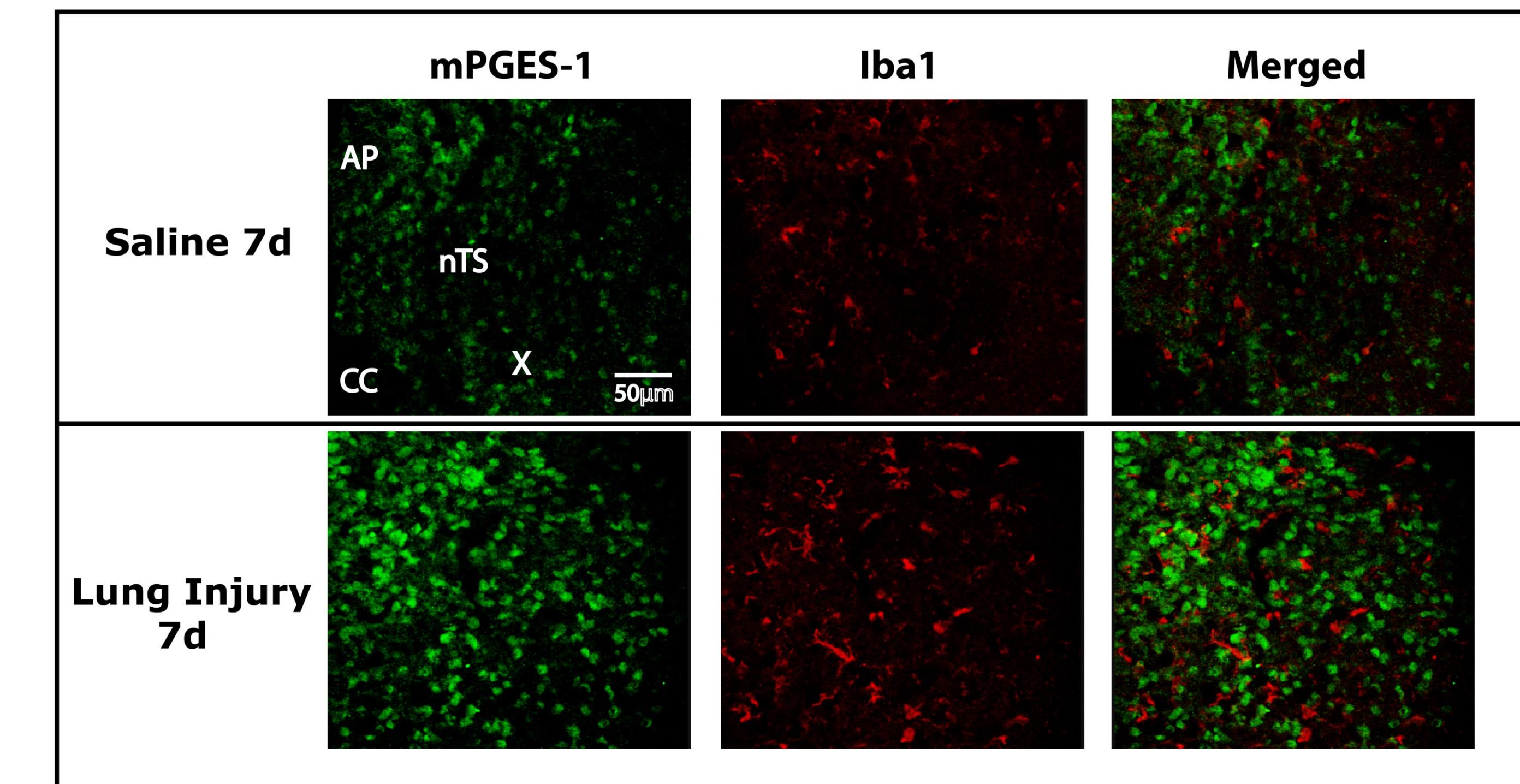


Figure 7: mPGES-1 is not co-localized with microglial marker Iba1 in the nTS at 7d post Bleo-induced lung injury. Dual label fluorescent IHC imaged via confocal microscopy reveals the presence of mPGES-1 in cell structures separate from microglia (N=6).

Conclusions:

- Pro-inflammatory cytokine expression remains increased in the dorsal vagal complex of the brainstem 7d after bleomycin-induced lung injury (Figure 1).
- Downstream targets of IL-1 β signaling, including COX-2 and mPGES-1, are also increased significantly (Figure 2).
- Immunoreactivity was greater in the nTS, AP, and DMV, which are centers of sensory integration, cardiorespiratory control, and inflammatory reflex.
- Enhanced HSP-70/72 immunoreactivity occurred in the nTS, AP, DMV, as well as the hypoglossal nucleus.
- Immunohistochemical staining for Iba1, a marker of microglial activity, shows no discernible change in microglial morphology or population density.
- Dual-label fluorescent immunohistochemistry indicates that brainstem microglia are not the primary site of mPGES-1 expression in the setting of bleomycin induced lung injury.
- We speculate that PGE₂ release in the nTS may occur via an astrocytic and neuronally mediated mechanism which, in the setting of lung injury, may contribute to changes in the ventilatory pattern.

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