Yes-associated Protein Induces Age-dependent Inflammatory Signaling in the Pulmonary Endothelium

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Abstract

Acute Lung Injury (ALI) causes the highly lethal Acute Respiratory Distress Syndrome (ARDS) in children and adults, for which therapy is lacking. Children with Pediatric ARDS (PARDS) have a mortality rate that is about half of adults with ARDS. Improved ALI measures can be reproduced in rodent models with juvenile animals, suggesting that physiologic differences may underlie these different outcomes. Here, we show that pneumonia-induced ALI caused inflammatory signaling in the endothelium of adult mice which depended on Yes-associated protein (YAP). This signaling was not present in 21-day-old weanling mice. Transcriptomic analysis of lung endothelial responses revealed nuclear factor kappa-B (NF-κB) as significantly increased with ALI in adult versus weanling mice. Blockade of YAP signaling protected against ALI and NF-κB nuclear translocation in adult mice. Our results demonstrate an important signaling cascade in the lung endothelium of adult mice that is not present in weanlings. We suggest other pathways may also exhibit age-dependent inflammatory signaling, which would have important implications for therapeutics in the adult and pediatric age groups.

Introduction

Acute lung injury (ALI) causes the Acute Respiratory Distress Syndrome (ARDS) and Pediatric ARDS (PARDS). Mortality rates in adult patients with ARDS are double those of PARDS patients (1–3). Preclinical studies in rodents show decreased lung injury measures in juvenile mice (4–6), suggesting that age-specific physiological differences underlie the different outcomes in ARDS and PARDS.

The Hippo pathway and its main effectors Yes-associated Protein (YAP) and PDZ-binding motif (TAZ) are vital for growth and differentiation in lung epithelium and in non-pulmonary organs (7–9). YAP is essential for the development of the endothelial barrier in the retina and the brain (10) and interacts with tight junction components in the lung epithelium (11). Recently, YAP was shown to protect against lung injury (12, 13). We hypothesized that YAP responses could underlie the differences in lung injury responses in juvenile and adult rodents.

Here, we instilled *Pseudomonas aeruginosa* into adult and weanling mice, and showed that lung injury measures are improved in weanlings. We show robust YAP protein expression in response to *P. aeruginosa* infection in adult mice, but not in weanlings. YAP knockdown prevented lung injury in adult mice, suggesting a causal role of YAP in our injury model. Knockdown of YAP decreased nuclear translocation of NF-κB in adult mice, suggesting that YAP contributes to pro-inflammatory signaling in the pulmonary endothelium of adult, but not juvenile mice.

Methods

Materials. For immunoblot, YAP mouse antibody (Cat#12395, 1:1000), YAP rabbit antibody (cat#14074, 1:1000), GAPDH mouse antibody (Cat#97166, 1:1000), and NF-κB p65 rabbit antibody (Cat#8242, 1:1000) were purchased from Cell Signaling Technology; HDAC1 mouse monoclonal antibody (Cat# 66085, 1:1000) was purchased from Proteintech; and actin rabbit antibody (cat# A2066, 1:1000) was purchased from Sigma-Aldrich. For lung cell isolation, CD31 rat monoclonal antibody clone MEC 13.3 (1:500, catalog 557355) was purchased from BD Biosciences. CD45 rat monoclonal antibody (1:600, catalog 14-0451-85), CD41 rat monoclonal antibody (1:500, catalog 14-0411-85), and CD326 rat

58 monoclonal antibody (1:500, catalog 14-5791-85) were purchased from Invitrogen. siRNA constructs
59 targeting Yap1 (siRNA ID #s76160) and Taz (siRNA ID #s211845) were purchased from ThermoFisher
60 Scientific. Male and female C57BL/6J mice were purchased from Jackson Laboratory
61 (RRID:IMSR_JAX:000664). *P. aeruginosa* strain K was obtained from Alice Prince (Columbia
62 University).

63 *Pseudomonas-induced lung injury. P. aeruginosa* was prepared as previously described (14). We
64 anesthetized mice and intranasally (i.n.) instilled 2.5 x 10^5 colony forming units (CFUs) *P. aeruginosa* at
65 exponential growth phase in 50µl sterile PBS (adult) or 20µl (weanling). 24 hours later, we anesthetized
66 the mice, performed a tracheotomy, and collected BAL as described (14) for cell count, protein
67 quantification, cytokine measurement, and determination of CFUs. We retained lungs after BAL for
68 downstream experiments.

69 *SpO_2 measurement*. For non-invasive O_2 saturation (SpO_2) measurements, we anesthetized mice and
70 removed the fur from right thigh. We placed a MouseOx® Pulse Oximeter sensor (Starr Life Sciences
71 Corp, Oakmont, PA) on the right femoral artery region and recorded SpO_2 after a 15-min stabilization
72 period.

73 *YAP RT-PCR.* We isolated RNA from whole lung using miRNeasy kit (Qiagen). We performed YAP RT-
74 PCR by using TaqMan Gene Expression Master Mix with QuantStudio™ 5 Real-Time PCR Instrument
75 (Thermo Fisher Scientific Applied Biosystems).

76 *YAP knockdown.* Anesthetized mice were in vivo transfected with YAP and TAZ siRNA (50µg in 150µL)
77 as in (14, 15) by tail vein injection 24 hours before *P. aeruginosa* instillation.

78 *Immunoblot.* Without freezing, we enriched lungs for cytoplasmic and nuclear fractions using NE-PER
79 Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific, cat #78833). Equal amounts of
80 protein were separated by SDS-PAGE, transferred onto nitrocellulose membrane, blocked in
81 StartingBlock Blocking Buffer (Thermo Scientific / Pierce, catalog 37543), and immunoblotting was
82 performed.

83 *Endothelial cell isolation.* Endothelial cells from C57BL/6J mice were isolated as described (16, 17) with
84 the following modifications. Lungs were digested for 15 min at 37°C in 0.38mg/ml Liberase (Roche) in
85 D-PBS + 0.1% albumin. We used a DynaMag-2 Magnet and Dynabeads Sheep Anti-Rat IgG (Invitrogen)
86 to deplete CD45+ and CD41+ cells, then positively enrich three times for CD31+ cells. These procedures
87 yielded ~10^5 cells per mouse lung. Bead isolation by this method yielded endothelial cells that were ~93%
88 pure and had <5% leukocyte contamination.

89 *Bulk RNA-seq.* RNA was isolated from freshly isolated endothelial cells bound to magnetic beads
90 (miRNeasy). RNA-seq was performed by Azenta. We aligned sequences to the GRCm39 mouse genome
91 using STAR aligner. We analyzed differential gene expression (DE) with EdgeR using a generalized linear
92 model with thresholds for significance set at <0.05 FDR (Benjamani-Hochenberg) and 1>|Log2(FC)|.

93 **Results**

94 *Differential lung injury responses in weanling and adult mice.*

95 To determine lung injury responses, we intranasally instilled weanling (P21-24) and adult (8-10 week old)
96 mice with *P. aeruginosa*. Twenty-four hours later, we assessed mice for lung injury by measurement of
97 bronchoalveolar lavage (BAL) protein, BAL cell count, BAL neutrophils, extra-vascular lung water, BAL
98 cytokines, and non-invasive measurement of O_2 saturation (SpO_2). Following *P. aeruginosa* instillation,
99 adult mice had an increase in BAL protein, BAL cell count, BAL neutrophils, EVLW, BAL Il-6 and BAL
100 TNF-α (**Fig 1A-E**), and a decrease in SpO_2 (**Fig 1F**) compared to weanling mice. Saline treatment did not
101 produce different responses in any lung injury measurements.

102 Given the difference in size of the mice (~10g vs. ~25g), we considered whether delivery of *P. aeruginosa*
103 could differ between the two age groups. In addition, decreased clearance of *P. aeruginosa* has been noted
104 in mice >20 days old (18). To assess delivery, we instilled the same number of CFUs in the same volume
105 to both age groups, and again in two different volumes. The lung injury results did not differ when the
106 volume was changed. To assess bacterial clearance, we measured BAL CFUs 24h after instillation and
107 noted no difference between the two age groups (**Supplemental Fig 1**). These findings suggest that a

similar number of CFUs was delivered in both ages and that bacterial clearance did not differ significantly between the age groups.

P. aeruginosa-induced changes in YAP protein expression differ with age

We determined changes in expression of YAP in response to *P. aeruginosa*-induced lung injury. Following BAL and a vascular wash, we enriched whole lungs from *P. aeruginosa* and saline treated mice into nuclear and cytoplasmic portions. We found that adult mice had robust increases in YAP protein expression in response to *P. aeruginosa* instillation in both cytoplasmic and nuclear fractions (**Fig 2A, B**). In contrast, weanling mice had decreased cytoplasm expression and no change in nuclear expression of YAP. Of note, there was no difference in YAP protein expression between age groups in saline-treated controls (**Supplemental Fig 2**), suggesting that baseline YAP expression does not differ between the two age groups. We assessed YAP mRNA expression with RT-PCR (**Supplemental Fig 3**) and noted that YAP mRNA expression in both weanling and adult mice decreased upon *P. aeruginosa*-induced injury, suggesting a post-transcriptional mechanism of increased YAP protein expression in adult mice.

P. aeruginosa-induced YAP protein increases occur in lung endothelial cells

Given the substantial increase in inflammatory cells in the BAL of adult versus weanling mice (**Fig 1B-C**), we considered whether leukocyte expression of YAP (19) contributed to YAP increases in adult mice. To determine the cell type in which YAP expression increased, we enriched for three fractions: lung endothelial cells, epithelial cells, and leukocytes plus platelets. We performed immunoblot for YAP in each of the enriched cell populations. We noted a substantial increase in YAP protein expression in endothelial cells (**Fig 2C**). There was no significant increase in YAP expression in epithelial cells (**Fig 2D**), and no YAP protein expression was detected in the leukocyte- and platelet-enriched fraction in PBS or *P. aeruginosa*-treated mice.

Vascular YAP mediates P. aeruginosa-induced lung injury in adult mice

131 Since YAP expression increases substantially in adult mice, but not in weanling mice, we hypothesized
132 that adult mice exhibit YAP-dependent lung injury responses that weanling mice lack. Thus, we targeted
133 endothelial YAP by tail vein injection of siRNA-targeted YAP and its paralog TAZ. Injection of YAP- and
134 TAZ-targeted siRNA decreased YAP expression in *P. aeruginosa* infected mice by ~80% (**Fig 3A**). We
135 assessed lung injury measures as in **Fig 1** and found that YAP knockdown prevented *P. aeruginosa*-
136 induced increases in BAL cell count, IL-6, and TNFα, as well as *P. aeruginosa*-induced decreases in SpO_2
137 (**Fig 3B-D**). This suggests that endothelial YAP expression is important for inflammatory responses in *P.*
138 *aeruginosa*-induced lung injury. Notably, YAP knockdown had no effect on BAL protein and EVLW,
139 suggesting that endothelial YAP may not be essential for barrier failure.

140 *YAP expression drives endothelial NF-κB responses in P. aeruginosa-infected adult mice*

141 To determine differences in *P. aeruginosa*-induced lung injury endothelial responses between weanling
142 and adult mice, we performed bulk RNA sequencing on freshly isolated endothelial cells from mice
143 treated with i.n. *P. aeruginosa* and control mice treated with i.n. PBS. In weanling mice, 2256 genes
144 showed increased expression in response to *P. aeruginosa*-induced injury and 2651 showed decreased
145 expression. In adult mice, 2732 genes increased expression and 3476 decreased. Of note, gene expression
146 differences between both age groups did not differ greatly at baseline (**Fig 4A**). However, transcriptomic
147 responses to lung injury differ vastly between age groups. This suggests substantial differences in lung
148 endothelial responses to inflammatory injury of weanling and adult mice.

149 We noted that there was no significant alteration in *yap1* gene expression with *P. aeruginosa* instillation in
150 either weanling or adult endothelia. We identified pathways that exhibited increased expression in adult
151 mice in response to *P. aeruginosa*-induced lung injury relative to weanling mice. The most significant
152 increases were found in TNFα signaling via NF-κB (**Fig 4B**). Although both weanling and adult
153 endothelial cells increased their expression of genes involved in this pathway, the adult endothelial
154 responses were increased relative to responses in weanling mice.

155 We considered whether YAP drives NF-κB nuclear translocation, leading to a more robust inflammatory
156 response in adult mice. First, we determined whether *P. aeruginosa* instillation drove NF-κB translocation
157 to the nucleus in adult mice. 24h after *P. aeruginosa* instillation, we enriched whole lung into nuclear and
158 cytoplasmic fractions. We noted a significant increase in NF-κB in the nuclear fraction and a decrease in
159 the cytoplasmic fraction (**Fig 4C**). In weanlings, there was an increase in NF-κB in the nuclear fraction,
160 but no significant difference in NF-κB quantity in the cytoplasmic fraction upon instillation of *P.*
161 *aeruginosa* (**Supplemental Fig 4**), suggesting that NF-κB nuclear translocation in response to *P.*
162 *aeruginosa* in weanlings may be decreased in comparison to adult mice. To determine whether the
163 increase in YAP expression in adult mice drove NF-κB translocation, we knocked down YAP expression
164 by vascular injection of siRNA as in **Fig 3A** and instilled *P. aeruginosa* into controls and siRNA-treated
165 mice 24h later. 24 hours after *P. aeruginosa* instillation, we measured NF-κB in the nuclear and
166 cytoplasmic fractions. We noted that YAP knockdown increased NF-κB protein expression in the
167 cytoplasm and decreased YAP expression in the nucleus (**Fig 4D**), suggesting that YAP mediated NF-κB
168 nuclear translocation.

169 **Discussion**

170 Our results differ from previous reports that endothelial YAP protects against lung injury (12, 13). YAP
171 appeared to inhibit NF-κB nuclear translocation by promoting degradation of tumor necrosis factor
172 receptor-associated factor 6 (TRAF-6) (13). In the epithelium, nuclear YAP may increase *ikba* gene
173 expression, inhibiting NF-κB nuclear translocation (20). Of note, in our transcriptomics dataset we noted
174 that expression of the *ikba* mouse homologue *nfkbia* was induced in both weanling and adult mice. This
175 suggests YAP-independent drivers of transcription that are active in weanling mice. In the above studies,
176 cell-specific YAP knockouts were used. It is possible that knockout of YAP leads to up-regulation of
177 alternative pathways (21). We suggest that our use of knockdown of YAP may more accurately reflect the
178 results of potential pharmacologic inhibition. The role of YAP in lung injury and repair is complex and
179 requires further study.

In neonatal mice in the early alveolar stage of lung development, NF-κB is known to play a protective role—ultimately promoting angiogenesis (17, 22–24). To our knowledge, this role has not been described in weanling mice in the late alveolar stage of lung development. However, it is possible that NF-κB nuclear translocation in weanling mice results in signaling that differs from canonical pro-inflammatory signaling in adults.

Our study has limitations. First, the signaling that leads to differential YAP expression between the weanling and adult age groups is unclear. Our RT-PCR (**Supplemental Fig 3**) and transcriptomics data suggest that the regulation is post-transcriptional, yet the drivers of these differences remain unknown. Second, our transcriptomics do not reveal the specific endothelial cell types that drive the inflammatory responses. We and others have identified the post-capillary venule as a primary location of inflammatory signaling (25–29, 14). However, a gene expression signature of this cell type to our knowledge remains elusive. Finally, the mechanism(s) by which YAP drives NF-κB nuclear translocation in adult mice remains unclear. This deserves further investigation.

Here, we show evidence for novel age-dependent inflammatory signaling in the pulmonary endothelium. Although pneumonia is a common cause of ARDS and PARDS, ALI signaling that leads to hypoxemia is orchestrated by the endothelium (15, 30). Our transcriptomics dataset suggests that there are multiple differences in inflammatory responses in the pulmonary endothelium between weanling and adult age groups, of which YAP-driven NF-κB is one example. Further investigation into age-dependent mechanisms of lung injury could aid in the tailoring of therapies across the lifespan.

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206 **Figure Legends**

207 **Figure 1. Responses to intranasal *Pseudomonas aeruginosa* in weanling and adult mice.** Analyses
208 were performed 24h after intranasal instillation of 2.5 x 10^5 CFU of *P. aeruginosa* or PBS. (**A-C, E**) BAL
209 was obtained by instillation of ice cold PBS intratracheally twice with ~80% recovery. (**A**) BAL protein
210 was determined by BCA assay. (**B-C**) BAL cell and neutrophil counts were determined with Vetscan
211 hematology analyzer. (**D**) ELVW was determined in separate experiments. (**E**) BAL cytokines were
212 measured by multiplex array (Eve Technologies). (**F**) SpO_2 was determined in anesthetized mice on a
213 warming blanket using a MouseOx® Pulse Oximeter. Data are shown as mean±SE. Number of replicates
214 are shown by dots (≥4 for all groups). *BAL*, bronchoalveolar lavage; *3wo*, 3 week-old mice;
215 *Pseudomonas*, *Pseudomonas aeruginosa; PMNs*, neutrophils; *EVLW*, extravascular lung water; *IL-6*,
216 interleukin-6; *TNF-α*, tumor necrosis factor α; SpO_2, non-invasive O_2 saturation. *p<0.05, ***p<0.001,
217 ****p<0.0001. Multiple comparisons were analyzed by two-way ANOVA; paired differences were
218 compared by unpaired 2-tailed t test.
219

220 **Figure 2. YAP expression responses to *P. aeruginosa*-induced lung injury in weanling and adult**
221 **mice.** Representative gels and scatter plots show immunoblotting and densitometry of enriched
222 cytoplasmic (**A**) and nuclear (**B**) fractions of whole lung, and enriched lung endothelial cells (**C**) and
223 epithelial cells (**D**). All animals were treated with i.n. PBS or 2.5 x 10^5 CFU of *P. aeruginosa* 24 hours
224 before lung removal. Antibodies used were YAP rabbit monoclonal, GAPDH mouse monoclonal, HDAC
225 mouse monoclonal, and actin rabbit polyclonal. Lanes were run on the same gel. *3wo*, 3 week-old mice;
226 n≥4 as indicated by dots. *n.s.*, not significant. *$p < 0.05$, ****$p<0.0001$. Differences between groups were
227 compared by t test.

228

229 **Figure 3. YAP mediates *P. aeruginosa*-induced lung injury in adult mice.** Mice were injected
230 intravenously with YAP- and TAZ-targeted siRNA or scRNA control 24h before *P. aeruginosa* intranasal
231 instillation. All mice were treated with *P. aeruginosa*. (**A**) Representative gel and scatter plots show
232 immunoblotting and densitometry of whole lung cytoplasmic fraction. Antibodies used were YAP rabbit
233 monoclonal and GAPDH mouse monoclonal. Note that there was high variability of lung YAP expression,
234 but statistical significance remained when the highest outlier from the scRNA-treated group was removed
235 from the analysis. (**B**) BAL cell counts were determined with Vetscan hematology analyzer. (**C**) BAL
236 cytokines were measured by multiplex array. (**D**) SpO_2 was determined using a MouseOx® Pulse
237 Oximeter. Number of replicates are shown by triangles (≥4 for all groups). *BAL*, bronchoalveolar lavage;
238 *3wo*, 3 week-old mice; *Pseudomonas*, *Pseudomonas aeruginosa; IL-6*, interleukin-6; *TNF-α*, tumor
239 necrosis factor α; SpO_2, non-invasive O_2 saturation. *p<0.05, **<0.01. Paired differences were compared
240 by t test.
241

242 **Figure 4. YAP expression drives endothelial NF-κB responses in *P. aeruginosa*-infected adult mice.**
243 (**A**) Principal component analysis of four treatment groups. PC1, treatment; PC2, age, orange, saline-
244 treated; blue, *P. aeruginosa*-treated. •, adult; **x**, weanling. (**B**) Top pathways with up-regulated genes in
245 adult mice relative to weanling mice in response to *P. aeruginosa* instillation. (**C**) Representative gel and
246 scatter plots show immunoblotting and densitometry of whole lung cytoplasmic and nuclear enriched
247 fractions (**C**) 24h after PBS or *P. aeruginosa* instillation and (**D**) following pretreatment with YAP siRNA
248 by tail vein injection and *P. aeruginosa* instillation. Antibodies used were NF-κB p65 rabbit monoclonal,
249 YAP rabbit monoclonal, HDAC mouse monoclonal, and actin rabbit polyclonal. Number of replicates are
250 shown by squares or triangles (≥3 for all groups). *NF-κB, nuclear factor-kappa B; scRNA, scrambled*
251 *siRNA; siRNA, YAP- and TAZ-directed siRNA*. *p<0.05, **p<0.01, ****p<0.0001. Paired differences
252 were compared by t test.
253

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Figure 1: Responses to intranasal *Pseudomonas aeruginosa* in weanling and adult mice

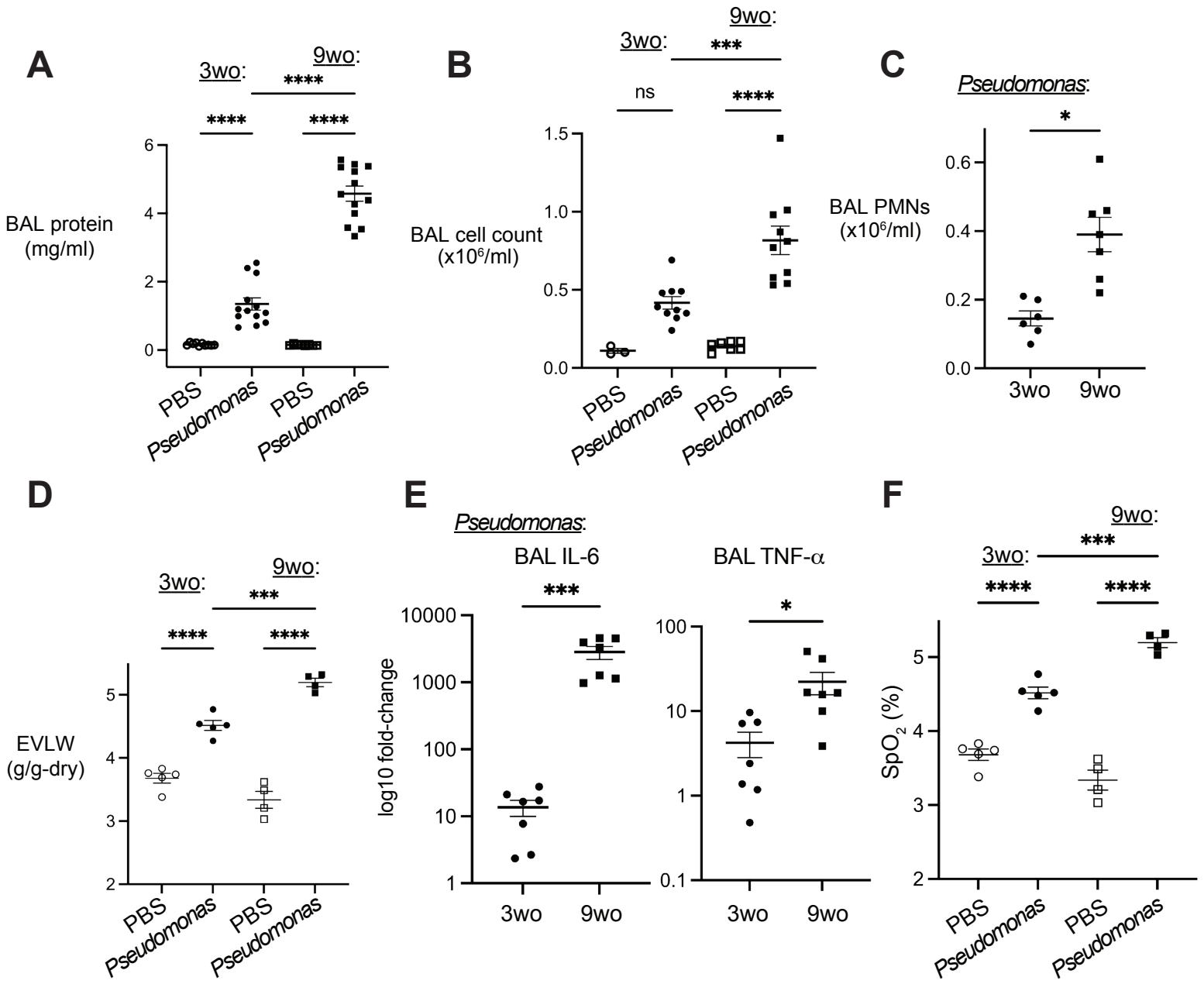

Figure 2: YAP expression responses to *P. aeruginosa*-induced lung injury in weanling and adult mice

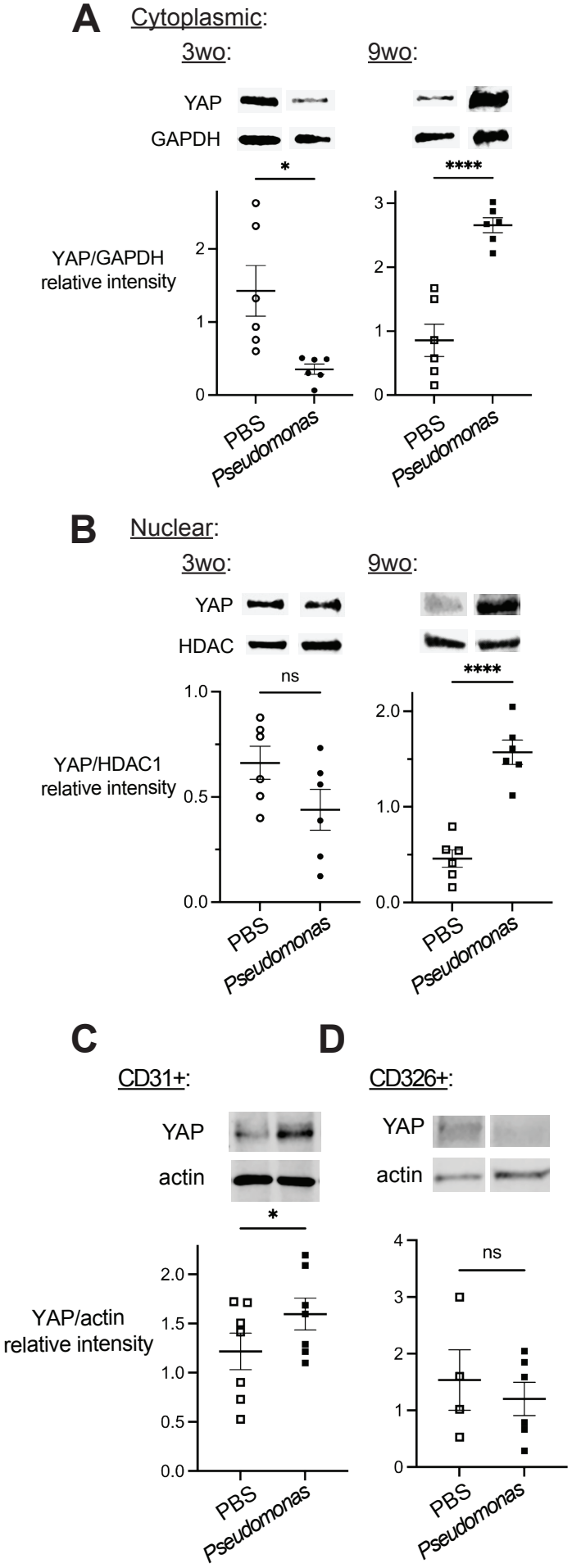

Figure 3: YAP mediates *P. aeruginosa*-induced lung injury in adult mice

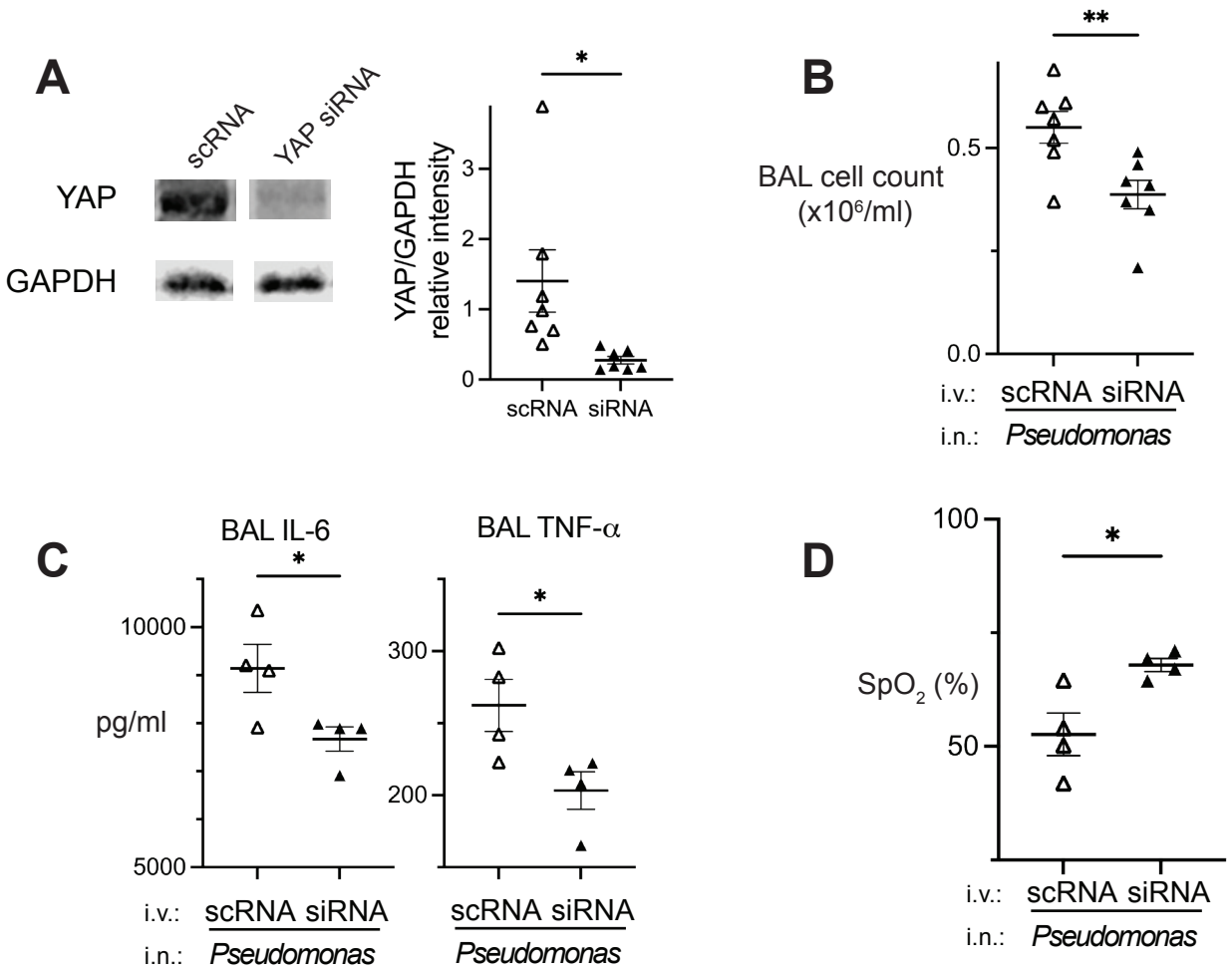
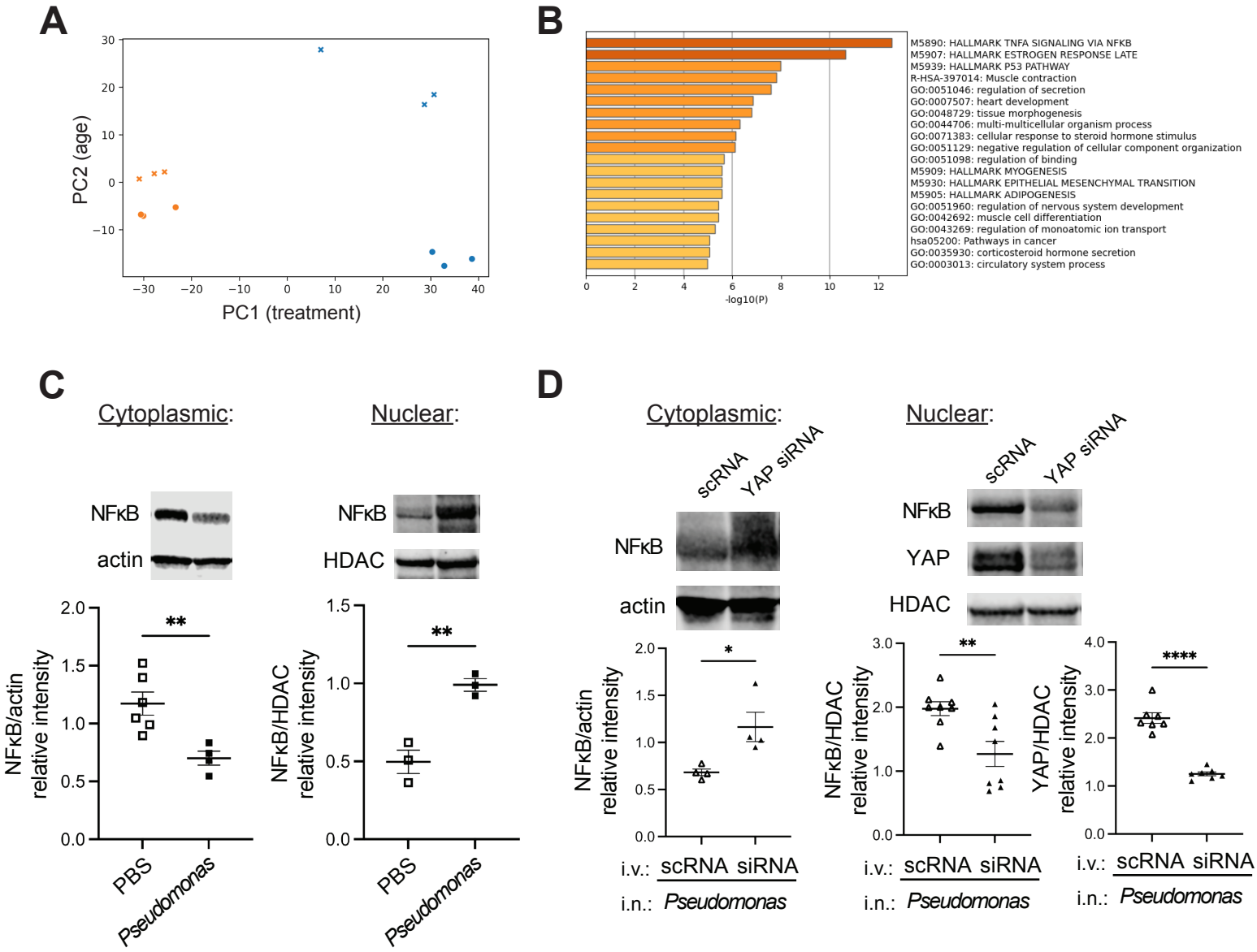
Figure 4: YAP expression drives endothelial NF-κB responses in *P. aeruginosa*-infected adult mice