

1 **Polarized Lung Inflammation and Tie2/Angiopoietin-Mediated Endothelial**  
2 **Dysfunction during Severe *Orientia tsutsugamushi* Infection**

3

4 **Running title: Pulmonary inflammatory mediators in severe scrub typhus**

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37 **Key Words:** *Orientia tsutsugamushi*, endothelial cell, Tie2, angiopoietin, macrophage

## 38 **Abstract**

39 *Orientia tsutsugamushi* infection can cause acute lung injury and high mortality in humans;  
40 however, the underlying mechanisms are unclear. Here, we tested a hypothesis that dysregulated  
41 pulmonary inflammation and Tie2-mediated endothelial malfunction contribute to lung damage.  
42 Using a murine model of lethal *O. tsutsugamushi* infection, we demonstrated pathological  
43 characteristics of vascular activation and tissue damage: 1) a significant increase of ICAM-1,  
44 VEGFR2, and angiopoietin-2 (Ang2) proteins in inflamed tissues and lung-derived endothelial  
45 cells (EC), 2) a progressive loss of endothelial quiescent and junction proteins (Ang1, VE-  
46 cadherin/CD144, occludin), and 3) a profound impairment of Tie2 receptor at the  
47 transcriptional and functional levels. *In vitro* infection of primary human EC cultures and serum  
48 Ang2 proteins in scrub typhus patients support our animal studies, implying endothelial  
49 dysfunction in severe scrub typhus. Flow cytometric analyses of lung-recovered cells further  
50 revealed that pulmonary macrophages (MΦ) were polarized toward an M1-like phenotype  
51 (CD80<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>) during the onset of disease and prior to host death, which  
52 correlated with the significant loss of CD31<sup>+</sup>CD45<sup>-</sup> ECs and M2-like  
53 (CD206<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>) cells. *In vitro* studies indicated extensive bacterial replication in  
54 M2-type, but not M1-type, MΦs, implying the protective and pathogenic roles of M1-skewed  
55 responses. This is the first detailed investigation of lung cellular immune responses during acute  
56 *O. tsutsugamushi* infection. It uncovers specific biomarkers for vascular dysfunction and M1-  
57 skewed inflammatory responses, highlighting future therapeutic research for the control of this  
58 neglected tropical disease.

59

## 60 **Author Summary**

61 Scrub typhus is a life-threatening disease, infecting an estimated one million people yearly.  
62 Acute lung injury is the most common clinical observation; however, its pathogenic biomarkers  
63 and mechanisms of progression remain unknown. Here, we used a lethal infection mouse model  
64 that parallels certain aspects of severe scrub typhus, primary human endothelial cell cultures, and  
65 patient sera to define pathogenic biomarkers following *Orientia tsutsugamushi* infections. We  
66 found a significant increase in the levels of endothelial activation/stress markers (angiopoietins  
67 and ICAM-1) in infected mouse lungs and in patient sera, but a progressive loss of endothelium-  
68 specific Tie2 receptor and junction proteins (VE-cadherin), at severe stages of disease. These  
69 signs of vasculature disruption positively correlated with the timing and magnitude of  
70 recruitment/activation of proinflammatory M $\Phi$  subsets in infected lungs. Bacterial growth *in*  
71 *vitro* was favored in M2-like, but not in M1-like, M $\Phi$ s. This study, for the first time, reveals  
72 endothelial malfunction and dysregulated inflammatory responses, suggesting potential  
73 therapeutic targets to ameliorate tissue damage and pathogenesis.

## 74 **Introduction**

75 Scrub typhus is a febrile and potentially lethal illness that infects an estimated one million  
76 individuals per year [1]. The disease is caused by infection with the bacterium, *Orientia*  
77 *tsutsugamushi*. Nearly a third of the human population lives in endemic areas, known as the  
78 “tsutsugamushi triangle”, although recent reports have identified scrub typhus in South America,  
79 which was previously believed to be free of scrub typhus [1, 2]. Within endemic areas, scrub  
80 typhus is reported to cause a substantial proportion (approximately 15-23%) of reported febrile  
81 illness [3, 4]. If left untreated, scrub typhus can manifest as interstitial pneumonia, myocardial  
82 and hepatic inflammation, and meningoencephalitis. [5]. Mild interstitial pneumonitis is typically  
83 the extent of pulmonary involvement during self-resolving or promptly treated scrub typhus.  
84 However, life-threatening pathologies can arise in severe cases, including lung hemorrhage,  
85 edema buildup, diffuse alveolar damage, and interstitial cellular infiltration [6]. Acute respiratory  
86 distress syndrome and lung damage are associated with high mortality and present in 6.75-25%  
87 of scrub typhus patients [5, 7]; however, there is no detailed investigation of the underlying  
88 mechanisms responsible for pulmonary endothelial dysfunction and inflammation.

89

90 Being an obligate intracellular bacterium, *O. tsutsugamushi* can infect a variety of host cells but  
91 primarily replicate in macrophages (MΦs)[8], dendritic cells [9], and endothelial cells (ECs) [6,  
92 8]. The bacteria enter host cells via the phagosome [10] or endosome [11], which they  
93 subsequently escape to begin replication in the cytoplasm. Infection-triggered cellular responses,  
94 including the activation of activator protein-1 (AP-1) and NF-κB pathways, the production of  
95 proinflammatory cytokines (IL-1β, TNF-α, IL-8/CXCL8), and the expression of distinct gene  
96 profiles, have been examined *in vitro* by using primary human umbilical vein endothelial cells

97 (HUVEC) [12], human epithelial/EC-like ECV304 cell line [13], human monocytes or MΦs  
98 [14]. Prolonged infection can result in EC death via apoptosis [15], but there is limited  
99 information on endothelial responses during the course of *O. tsutsugamushi* infection [16].  
100 Sublethal *O. tsutsugamushi* infection studies in outbred Swiss CD-1 mice [17], as well as clinical  
101 studies of human patients [18], have shown significant elevation of endothelial activation  
102 markers (ICAM-1, VCAM-1, E-Selectin, etc.) in the serum of infected individuals; however,  
103 endothelium-focused analyses during *in vivo* infection remain largely unexplored. Several  
104 studies have characterized the response and activation of MΦs during *O. tsutsugamushi* infection  
105 [19, 20]. *In vitro* and *ex vivo* experiments have shown that human monocytes/MΦs in the  
106 circulation adopt an inflammatory “M1” type transcriptional profile after *O. tsutsugamushi*  
107 infection, although little is known regarding tissue specific macrophages or the presence of  
108 alternatively activated “M2”-type macrophages [21].

109  
110 The delicate balance of EC quiescence and activation is crucial during systemic infection. While  
111 EC activation promotes adherence and recruitment of innate and adaptive immune cells for  
112 pathogen clearance, prolonged activation can lead to EC cytotoxicity, impaired barrier function,  
113 and host mortality [22]. One of the critical mechanisms to control EC activation status and  
114 cellular function is through competitive interactions between angiopoietin-1 (Ang1) and Ang2  
115 ligands with their receptor, Tie2, a protein tyrosine kinase that is predominately expressed on  
116 ECs in humans and mice. Tie2 activation and phosphorylation via binding with Ang1 (produced  
117 by pericytes and platelets [23]) promote EC quiescence, which limits leukocyte adhesion and  
118 maintains EC survival and vascular barrier integrity [24]. Infection- or inflammation-triggered  
119 release of Ang2 (normally stored within the Weibel-Palade bodies in ECs [25, 26]) can compete

120 with Ang1 binding Tie2 to antagonize its function [27]. Inhibition of Tie2 signaling via Ang2  
121 binding stimulates leukocyte adhesion, vascular barrier destabilization, and inflammation [28,  
122 29]. Thus, Ang2/Ang1 expression ratios and Tie2 activation status are important biomarkers for  
123 the pathogenesis of systemic infection, such as severe sepsis and malaria [30, 31].

124

125 To investigate endothelial alterations during severe *O. tsutsugamushi* infection, we have recently  
126 developed a lethal intravenous *O. tsutsugamushi* infection model in C57BL/6 mice [32-34],  
127 which parallels aspects of severe scrub typhus in humans. In our lethal models, bacterial loads in  
128 both the spleen and liver reached peak levels around or shortly after the onset of disease (days 6-  
129 8 post-infection), and are reduced significantly by days 10-12. In contrast, lung bacterial loads  
130 remain elevated throughout infection [35]. All mice expire by days 12-13, suggesting unknown  
131 mechanisms of pathogenesis are at work during late infection [32, 35]. Given that the lungs are a  
132 major organ for *O. tsutsugamushi* infection in humans and in different animal models, and that  
133 elevated ratios of *ANG2/ANG1* transcripts are pathological hallmarks in lethal infection models  
134 [32, 35], we hypothesize that dysregulated pulmonary inflammation and Tie2/Ang2-mediated  
135 endothelial dysfunction contribute to disease pathogenesis at late stages of *O. tsutsugamushi*  
136 infection.

137

138 In this study, we utilized a lethal infection model in C57BL/6 mice, *in vitro* infection systems  
139 (human EC cultures, and bone marrow-derived MΦ subsets), as well as sera from scrub typhus  
140 patients to reveal positive correlations between vascular dysfunction, activation of innate  
141 immune cells, and disease progression. We focused on two cellular subsets known to be sites of  
142 *O. tsutsugamushi* replication, ECs and MΦs, to characterize their activation and polarization *in*

143 *vivo*. To the best of our knowledge, this is the first report to delineate MΦ subsets in inflamed  
144 lungs and their positive correlation with Tie2 malfunction during late stages of severe infection.  
145



## 146 **Results**

147

### 148 **Pulmonary EC activation and tight junction disruption during infection in mice**

149 Given that the lung is a major site of *O. tsutsugamushi* infection in humans and animal models  
150 [6, 32] and that EC activation and disruption of vascular barrier integrity are principal steps for  
151 acute lung injury in sepsis and pneumonia models [36], we sought to investigate pulmonary EC  
152 activation in C57BL/6 mice following i.v. inoculation with a lethal dose of *O.*  
153 *tsutsugamushi* Karp ( $\sim 1.325 \times 10^6$  viable bacteria in 200  $\mu$ l of PBS). Inoculation via this route  
154 establishes bacterial replication in the lungs accompanied by interstitial pneumonitis and alveolar  
155 thickening (S1A Fig, [35]). Immunofluorescent staining of frozen lung sections revealed  
156 increased ICAM-1-positive (green) vascular staining on days 2 (D2) to 9, as well as a close  
157 association of bacterial (red) with activated endothelium at D9 (Fig 1A, boxed area, Fig 1C). To  
158 examine endothelial structure and adherens junctions, we co-stained lung sections with GSL I-B<sub>4</sub>  
159 lectin (specific for  $\alpha$ -galactose residues known to be enriched on the surface of EC) and anti-VE-  
160 cadherin/CD144 (an adherens junction protein, red), as described in our previous report for  
161 neuroinflammation [34]. The VE-cadherin staining was intense and homogenous in the control  
162 tissues, but markedly reduced in D2 samples; VE-cadherin staining was nearly absent in some  
163 foci of D6 and D9 samples (Fig 1B-C), implying the reduction of junction proteins. In  
164 conjunction with the endothelial junction proteins, we co-stained the epithelial junction protein,  
165 occludin, and GSL I-B<sub>4</sub> lectin. Consistently, we found a progressive loss of occludin staining on  
166 the bronchial epithelium during the infection and a near absence of staining in D9 samples (S1B  
167 Fig). These data suggest a progressive and severe loss of vascular barrier integrity in the infected  
168 lungs, especially at late stages of acute infection prior to host death (D9) [32].

169

170 To support our immunofluorescent results, we prepared single-cell suspensions from mouse lung  
171 tissues and used flow cytometry to examine the frequency of recovered CD31<sup>+</sup>CD45<sup>-</sup> ECs and  
172 their surface ICAM-1 expression. We found that compared with the mock controls, infected lung  
173 tissues contained a significant increase in the frequencies of ICAM-1<sup>+</sup>CD31<sup>+</sup>CD45<sup>-</sup> ECs at D6  
174 and D9, respectively ( $p < 0.001$ ,  $p < 0.01$ , Fig 1D), while there were approximately 5-folds  
175 reduction in the frequencies of total ECs at D6 and D9 ( $p < 0.001$ , S1C Fig). We also examined  
176 EC expression of vascular endothelial growth factor receptor 2 (VEGFR2), a critical factor  
177 controlling vascular permeability and barrier function [37]. Compared with the mock controls,  
178 there was an approximately 6-fold increase in the frequencies of VEGFR2<sup>+</sup>CD31<sup>+</sup>CD45<sup>-</sup> ECs in  
179 the lung tissues at D6 and D9 (Fig 1D). These flow cytometry data reinforced the  
180 immunofluorescent results, indicating marked endothelial activation and damage at D6 (the onset  
181 of disease) and D9 (prior to host demise). To validate these findings in mice, we infected primary  
182 HUVEC cultures with different doses of *O. tsutsugamushi* (3 and 10 MOI) and found a dose-  
183 dependent increase in *ICAM1* and *IL8/CXCL8* transcripts at 24 h post-infection (S1D Fig).  
184 Collectively, these data indicate infection-triggered endothelial stress and activation,  
185 accompanied by progressive vascular damage and tight junction disruption during the course of  
186 infection.

187

### 188 **Alterations in the angiopoietin-Tie2 system during *Orientia* infection**

189 Currently, there are no detailed *in vivo* studies to define molecular mechanisms underlying *O.*  
190 *tsutsugamushi* infection-associated vascular damage. For other severe and systemic infections  
191 caused by bacteria or viruses, alterations in angiopoietin proteins or their functional Tie2

192 receptor is considered as one of the key mechanisms for vascular dysfunction [38, 39] . Given  
193 our previous findings of elevated *ANG2/ANG1* mRNA ratios in mice with severe scrub typhus  
194 and in *O. tsutsugamushi*-infected HUVECs [33], we speculated that impairment in Tie2 function  
195 occurs in severe scrub typhus [16]. To test this hypothesis, we examined Ang1, Ang2, and Tie2  
196 protein levels in the lung tissues via immunofluorescent staining (Figs 2-3). While the mock  
197 controls contained relatively high levels of Ang1 (green), with relatively low levels of Ang2  
198 (red), we found a modest decrease in Ang1-positive staining but a steady increase in Ang2  
199 staining, during the course of infection (Fig 2A). These IFA results were quantified,  
200 demonstrating the significant decrease of Ang1 and a significant increase of Ang2 expression  
201 during *O. tsutsugamushi* infection that correlated with disease progression (Fig 2B). To validate  
202 our findings mouse models, we measured scrub typhus patient sera via specific ELISA assays  
203 and found a statistically significant increase in circulating Ang2 levels, which correlated with  
204 their *O. tsutsugamushi*-specific antibody titers ( $p < 0.05$  and  $p < 0.01$ , comparing IFA titers of  
205 1:640 and 1:1280 with the control subjects, Fig 2C). These human data support our findings  
206 obtained from mouse tissues, indicating the potential utility of serum Ang2 levels as a molecular  
207 biomarker of scrub typhus severity.

208

209 IFA staining of the Ang1/2 receptor, Tie2, revealed clear Tie2 staining in mock infected controls,  
210 however, positive Tie2 staining was nearly absent in some foci of D6 and D9 samples (Fig 3A).  
211 To validate these findings, we used Western blot analyses of lung tissues and confirmed a  
212 striking reduction of phosphorylated Tie2 (pTie2) and total Tie2 levels at both D6 and D9, as  
213 compared with either the mock and D2 samples (Fig 3B), implying impairments at the  
214 translational and functional levels. The qRT-PCR analyses further validated a statistically

215 significant decrease in *TIE2* mRNA levels in the lungs at D6 and D10 ( $p < 0.01$ , compared with  
216 the mock controls, Fig 3C), implying impairment at the transcriptional level. These data, together  
217 with our previous studies [32, 33], indicate that marked Ang2 production, accompanied with  
218 severe impairment in the Tie2 functions, are pathogenic mechanisms of severe vascular damage  
219 in *O. tsutsugamushi* infection.

220

### 221 **M1-like responses in the lungs of infected mice**

222 Having documented progressive endothelial damage and alterations in endothelium-specific  
223 biomarkers following *O. tsutsugamushi* infection (Figs 1 and 2), we then examined the timing  
224 and magnitude of leukocyte recruitment and activation. Although some reports described  
225 leukocyte involvement in *O. tsutsugamushi*-infected mouse spleen and brain [34, 40], there are  
226 no detailed studies of innate immune responses in infected lungs. Using immunofluorescent  
227 staining, we found that CD45<sup>+</sup> leukocytes and CD3<sup>+</sup> T cells were accumulated around Ang2-  
228 positive foci in the lungs at D6 and D10, and that CD45-Ang2 or CD3-Ang2 co-stained foci  
229 were readily detectable at D10 (yellow, S2A Fig). Flow cytometric analyses revealed a 20-fold  
230 increase in total numbers of CD4<sup>+</sup> T cells at D10, but a statistically significant decrease in  
231 percentages of these cells at D6 and D10, respectively ( $p < 0.0001$ , compared with mock  
232 controls, S2B Fig). In contrast, there was a 50-fold increase in total numbers and 2.3-fold  
233 increase in percentages of CD8<sup>+</sup> T cells at D10 (S2C Fig). These findings were consistent with  
234 the known importance of CD8<sup>+</sup> T cells during *O. tsutsugamushi* infection in mice [40, 41].

235

236 Monocytes and MΦs are particularly noteworthy leukocytes during *O. tsutsugamushi* infection,  
237 as they can act as a target for bacterial replication and a propagator of the inflammatory response

238 [8, 14], possibly playing a role in *O. tsutsugamushi* dissemination from skin lesions [9, 42].  
239 While *in vitro* infection predominantly drives human monocytes/MΦs to M1-like transcription  
240 programs [9, 42], our current knowledge on *O. tsutsugamushi*-MΦ interactions in the lungs is  
241 still limited. Using IFA staining, we observed co-localization of bacteria (green) with IBA-1<sup>+</sup>  
242 MΦs (red) in mouse lungs (S3A Fig). To define monocyte/MΦ responses, we applied reported  
243 protocols and gating strategies for flow cytometric analysis of mouse lung monocyte/MΦ subsets  
244 [43] (Fig 4A). Compared with the mock controls, D6 and D9 samples had 4- to 5-fold increases  
245 in the frequency of CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup> alveolar/interstitial monocytes/MΦs, as well as 6- and  
246 14-fold increases in total cell numbers, respectively ( $p < 0.01$  and  $p < 0.001$ , Fig 4B). Of note,  
247 nearly all (~97%) pulmonary MΦs displayed an M1-like phenotype  
248 (CD80<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>, Fig 4C) at D9. In contrast, while the mock and D2 lung samples  
249 contained ~3.2% of M2-like (CD206<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>) cells, these cells were barely  
250 detectable at D6 or D9 (Fig 4D). These data suggest extensive recruitment and/or activation of  
251 M1-like cells, but marked loss and/or suppression of M2-like cells, during the progression of  
252 disease. Likewise, lung qRT-PCR assays confirmed a significant up-regulation of M1 markers  
253 (*IFN $\gamma$* , *FPR2*, *CD38*, and *NOS2*), but not M2 markers (*CD206*, *EGR2*), at D9 (Fig 5A and B).  
254 While the *IL10* up-expression was previously reported by our lab and other groups [44], we also  
255 detected an increased expression of *ARGINASE1* (Fig 5B), a marker known for M2 polarization  
256 and the growth of other intracellular pathogens [45]. Together with data shown in Fig 4, we  
257 concluded that at the onset of disease and beyond, *O. tsutsugamushi* infection preferentially  
258 stimulated pro-inflammatory innate responses in M1-like monocytes/MΦs, which correlate with  
259 the onset of vascular damage (Figs 1-3).

260

## 261 **MΦ polarization in favor of *Orientia* replication *in vitro***

262 Because we had demonstrated differential monocyte/MΦ responses *in vivo*, we asked how  
263 MΦ polarization might influence intracellular growth of the bacteria. We generated bone  
264 marrow-derived MΦs from naïve C57BL/6 mice, polarized cells via pretreatment with LPS (100  
265 ng/ml) or recombinant IL-4 (rIL-4, 10 ng/ml) for 24 h, infected cells with *O. tsutsugamushi*  
266 (MOI 5), and measured bacterial loads at different time points. Flow cytometry and gene profile  
267 analyses of primed but uninfected cells confirmed their corresponding polarization to either  
268 classically activated M1 or alternatively activated M2 phenotypes (S3B and C Fig), as  
269 documented by others [46]. At 48 h post-infection, IL-4-primed M2 cells contained significantly  
270 increased loads of bacteria (judged by the copy number of *Orientia* 47-kDa gene) than LPS-  
271 primed M1 cells ( $p < 0.01$ , Fig 6A). At 72 h post-infection, M2 cells contained 10-fold more  
272 bacteria than M1 cells (Fig 6A), with extensive accumulation of bacteria (green) within IBA-1-  
273 positive MΦs (red, Fig 6B).

274

## 275 **Discussion**

276 Despite being an important emerging infectious disease, detailed immunological studies of scrub  
277 typhus patient samples or *O. tsutsugamushi*-infected animal tissues during the course of disease  
278 are scarce. In this study, we focused on ECs and monocytes/MΦs in the lungs of lethally infected  
279 mice to examine the activation of these cellular subsets known to be important cellular targets of  
280 *O. tsutsugamushi* *in vivo*. Our findings revealed important parameters and cell-specific  
281 alterations associated with acute lung injury and pathogenesis. The endothelium in infected lungs  
282 presented progressive Tie2 malfunction, increased Ang2 and ICAM-1 expression and pro-  
283 inflammatory MΦs at the onset of disease and severe stages of infection. Since lung damage and

284 vascular malfunction are hallmarks of scrub typhus severity in patients [6], a better  
285 understanding of pathogenesis associated with acute lung injury is important for disease control  
286 and management.

287

288 The molecular characteristics of endothelial alterations during *O. tsutsugamushi* infection *in vivo*  
289 have not been explored. The present study provided evidence for the mechanisms underlying  
290 pulmonary injury and vascular dysfunction during *O. tsutsugamushi* infection. First, the timing  
291 of ICAM-1 and VEGFR2 expression on the surface of lung-derived CD31<sup>+</sup>CD45<sup>-</sup> ECs was  
292 concurrent with the appearance of signs of vascular injury and decrease in cell junction proteins  
293 (Fig 1 and S1 Fig). Since ICAM-1 promotes circulating immune cells to bind to the endothelium  
294 and extravasate into inflamed tissues [47], increased ICAM-1 surface expression likely  
295 contributed to immune cell influx into the infected mouse lungs (Fig 5, S2 Fig). Our observed  
296 *ICAM1* and *IL-8/CXCL8* up-regulation in infected human EC cultures (S1C Fig) were consistent  
297 with other reported studies of scrub typhus patients [48]. VEGFR2 is known to be the primary  
298 receptor for vascular endothelial growth factor (VEGF) in the endothelium, and the  
299 VEGF/VEGFR2 axis regulates microvascular permeability via interacting with VE-cadherin and  
300 tight junction proteins [49, 50]. In our hands, we consistently detected a marked increase in  
301 VEGFR2 on the surface of infected ECs, but a significant reduction of VE-cadherin (adhesion  
302 junctions) in the lungs at D6 and D9. While it is unclear whether increased VEGFR2 expression  
303 is directly linked to diminished VE-cadherin expression in infected lungs, our data suggest  
304 compromised endothelial barrier integrity during severe *O. tsutsugamushi* infection in the lungs.

305

306 Second, a notable reduction of Tie2 proteins was concurrent with significant Ang2 production  
307 and/or release at the severe stages of infection (Figs 2-3). To date, there are no reports for Tie2  
308 expression levels in scrub typhus patients or animal models, although our group previously  
309 showed increased *ANG2/ANG1* transcript ratios in *O. tsutsugamushi*-infected human EC cultures  
310 and mouse tissues [32]. Our findings of significant and progressive reduction in Tie2 protein and  
311 its mRNA levels, as well as functional pTie2 level, in the lungs of lethally infection mice are  
312 important from basic research and clinical points of view. It is known that the Ang1/Tie2 axis is  
313 essential for vascular remodeling and endothelial cell stabilization, as either knockout Ang1 or  
314 Tie2 in mice is embryonic lethal [51, 52]. Given the critical function of Tie2 receptor in vascular  
315 physiology and integrity, it will be important to further examine whether our observed reduction  
316 in Ang1 and Tie2 is due to direct endothelial damage or signaling from nearby pericytes and  
317 recruited immune cells. Research in these areas would be of great value, as angiopoietin- or  
318 Tie2-targeted therapies have been evaluated as alternative treatment strategies for severe sepsis  
319 [53, 54], severe dengue [55], and in cerebral malaria [38] infection models to restore endothelial  
320 quiescence during infection. Our clinical observation that increased serum Ang2 in human scrub  
321 typhus patients correlates with *O. tsutsugamushi*-specific antibody titers demonstrates the utility  
322 of Ang2 as a pathogenic biomarker, and highlights the potential of use Ang2- or Tie2-targeted  
323 therapies for severe scrub typhus, as in patients with severe sepsis [29, 30] and malaria [56].

324

325 Monocytes/MΦs play important roles in infection with *O. tsutsugamushi* and other closely  
326 related *Rickettsia* species [19, 57]. Previous findings [19] are consistent with our observation of  
327 an increased accumulation of CD11b<sup>+</sup>Ly6G<sup>-</sup> MΦs/monocytes and the close association of IBA-1<sup>+</sup>  
328 phagocytes with *Orientia* in the lungs at D6 and D9. Yet, our findings of selective



329 recruitment/expansion of M1-skewed MΦs/monocytes, and the suppression of M2 cell  
330 activation, in the infected mouse lungs were particularly novel and important. This is the first *in*  
331 *vivo* evidence for M1-polarized MΦ responses in *O. tsutsugamushi*-infected mouse lungs, which  
332 was consistent with previous *in vitro* and *ex vivo* studies for M1-skewed gene transcription  
333 programs in *O. tsutsugamushi*-infected human monocytes and MΦs [21, 42]. At present, it is still  
334 unclear whether the predominately M1 MΦ population we observed contributes to the killing of  
335 *O. tsutsugamushi* and the damage of vascular tissues, as seen for protective versus pathogenic  
336 roles in other lung infection models [58]. Given the reports that anti-Ang2 antibody treatment  
337 during pulmonary bacterial infection can decrease MΦ recruitment and inflammation [26], and  
338 that signaling via Tie2 on monocytes/MΦs can promote a proinflammatory profile [59], it will be  
339 interesting to determine how Ang2/Ang1 signaling on both endothelial cells and MΦs promote  
340 cellular recruitment and activation during *O. tsutsugamushi* infection.

341  
342 Conditions that promote the killing or growth of *O. tsutsugamushi* remain unclear, in part due to  
343 difficulties in bacterial cultivation, genetic modification, or visualization for studying the host-  
344 bacterium interactions [60]. Currently, there are no available reports or data for the phenotype of  
345 *Orientia*-carrying MΦ subsets or the roles of arginase-1 *in vivo*. Our *in vitro* comparative studies  
346 revealed limited bacterial replication in LPS-primed M1-like MΦs (Fig 6), supporting a previous  
347 report for the role of NOS2-mediated mechanisms in the control of *O. tsutsugamushi* Karp strain  
348 [19]. Yet, our *in vitro* findings were contradictory to another reported study, in which NO-  
349 enhanced the growth of *O. tsutsugamushi* Ikeda bacteria was observed in LPS-activated RAW  
350 264.7 murine macrophages at days 6 to 8 post-infection [20]. While the use of different bacterial  
351 strains, MΦ sources, and examination times may account for these discrepancies, our study calls

352 for careful examination of the intracellular niche for the replication of these obligate intracellular  
353 bacteria under different or physiological conditions. For bone marrow-derived MΦs, we found  
354 comparable bacterial loads in LPS- and IL-4-primed cells at 3 h, implying similar attachment and  
355 invasion of the bacteria under these two treatments. However, IL-4-primed M2 cells contained  
356 10-fold more *O. tsutsugamushi* than LPS-primed M1 cells at 72 h. At present, our data did not  
357 exclude the possibility of bacterial growth in LPS-primed M1 cells after prolonged time *in vitro*,  
358 or in M1- vs. M2-like MΦs in mouse lungs or other organs. Given the recent report that specific  
359 MΦ responses, such as miR-155 and IL-10 production, correlate with prevention of cytokine  
360 storm in severe *O. tsutsugamushi* infection [61], it will be important to further examine whether  
361 the strong type-1 inflammatory responses *in vivo* [32] and M1-skewed responses (Fig 5) are  
362 responsible for marked decrease in M2 MΦs in the lungs. The use of transgenic mouse strains  
363 for tracking MΦ subsets would also help reveal whether *O. tsutsugamushi* bacteria preferentially  
364 replicate within M2 MΦs *in vitro* or contribute to the impairment of type 2 immune responses.

365

366 While the mouse model used here allows us to examine host-bacterium interactions and immune  
367 alterations, this model has some intrinsic limitations, as it bypasses bacterial dissemination from  
368 the local/skin sites. Nevertheless, this model has several advantages over self-limited infection  
369 models following the subcutaneous or intradermal inoculation of the bacteria via needles into  
370 inbred strains of mice [19, 62], for the examination of innate immune responses in visceral  
371 organs. Compared to a lethal infection initiated by feeding *O. tsutsugamushi*-infected mites on  
372 outbred mice, a technically challenging model with high variability [63], our model provides  
373 more consistent results and lethality, permitting the analysis of a given host molecule using gene-  
374 targeted knockouts on the C57BL/6 background. More importantly, our lethal model mimics

375 certain pathological aspects of severe scrub typhus observed in humans, uncovering tissue-  
376 specific immune alterations that have never been described previously. For example, our findings  
377 of elevated Ang2 proteins in *O. tsutsugamushi*-infected lungs and increased *ANG2* expression in  
378 multiple organs [32] are consistent with clinical studies of scrub typhus patients (Fig 2), which  
379 supports the potential for monitoring serum Ang2 levels as an indicator of disease severity and  
380 treatment outcome.

381

382 In summary, this study has revealed new insights into immune dysregulation and pathogenesis of  
383 severe scrub typhus. Through comprehensive analyses of *O. tsutsugamushi*-infected mouse lung  
384 tissues, we have provided the first evidence for the production of endothelial destabilizing factors  
385 and *in vivo* polarization of lung recruited MΦs. Our findings of polarized M1-like responses in  
386 the lungs at late stages of disease argue for immune-based restriction of bacterial replication as  
387 well as immunopathogenesis. While the molecular mechanisms underlying host-bacterium  
388 interaction and immune dysregulation remains unclear at this stage, it is conceivable that serum  
389 and tissue Ang2 levels would be a molecular biomarker for severe scrub typhus and a potential  
390 therapeutic target for treatment. A better understanding of infection- versus immune-mediated  
391 dysregulation will help design treatment strategies for severe scrub typhus cases.

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## 398 **Materials and Methods**

399

### 400 **Mouse infection and ethics statement**

401 Female C57BL/6 mice were purchased from Envigo (Huntingdon, United Kingdom), maintained  
402 under specific pathogen-free conditions and used at 6-9 weeks of age, following protocols  
403 approved by the Institutional Animal Care and Use Committee (protocols # 9007082B and  
404 1302003) at the University of Texas Medical Branch (UTMB) in Galveston, TX. All mouse  
405 infection studies were performed in the ABSL3 facility in the Galveston National Laboratory  
406 located at UTMB; all tissue processing and analysis procedures were performed in the BSL3 or  
407 BSL2 facilities. All procedures were approved by the Institutional Biosafety Committee, in  
408 accordance with Guidelines for Biosafety in Microbiological and Biomedical Laboratories.  
409 UTMB operates to comply with the USDA Animal Welfare Act (Public Law 89-544), the Health  
410 Research Extension Act of 1985 (Public Law 99-158), the Public Health Service Policy on  
411 Humane Care and Use of Laboratory Animals, and the NAS Guide for the Care and Use of  
412 Laboratory Animals (ISBN-13). UTMB is a registered Research Facility under the Animal  
413 Welfare Act, and has a current assurance on file with the Office of Laboratory Animal Welfare,  
414 in compliance with NIH Policy.

415

416 *O. tsutsugamushi* Karp strain was used herein; all infection studies were performed with the  
417 same bacterial stocks prepared from Vero cell infection, for which infectious organisms were  
418 quantified via a qPCR viability assay [35, 64]. Mice were inoculated intravenously (i.v.)  
419 with  $\sim 1.325 \times 10^6$  viable bacteria (a lethal dose, 200  $\mu$ l) or PBS and monitored daily for weight

420 loss and signs of disease. In most cases, tissue samples (4-5 mice/group) were collected at 2, 6,  
421 and 9 (or 10) days post-infection and inactivated for immediate or subsequent analyses.

422

423 Ethical approval for human samples used in this work was granted by the Institutional Review  
424 Board of both Seoul National University Hospital (IRB NO 1603-136-751) and Chungnam  
425 National University Hospital (IRB NO 2014-12-006). All patients and healthy volunteers  
426 provided written informed consent prior to sample collection.

427

### 428 **Immunofluorescence microscopy and quantification**

429 Mouse lung tissues were processed for immunofluorescent staining, as in our previous report  
430 [34]. Briefly, 6- $\mu$ m frozen sections were blocked and incubated with the following rat or rabbit  
431 anti-mouse antibodies (1:200, purchased from Abcam, Cambridge, MA, USA, unless specified):  
432 anti-ICAM1, anti-Ang1, anti-Ang2 (R&D Systems/Biotechne, McKinley Place NE, Minnesota),  
433 anti-Tie2, anti-VE-cadherin (adherence junctions), anti-occludin (epithelial tight junctions), anti-  
434 IBA-1 (ionised calcium binding adapter molecule-1, a M $\Phi$  marker), anti-CD45 (BD Bioscience,  
435 San Jose, CA, USA), or anti-Ang2 (R&D Systems). Staining endothelium in sections was done  
436 with FITC-conjugated *Griffonia Simplicifolia* lectin I (1:100, GSL I-B<sub>4</sub> lectin, Vector Lab,  
437 Burlingame, CA, USA). Bacteria was stained in various sections using rabbit anti-*O.*  
438 *tsutsugamushi* Karp serum (1:500) [35]. Bound antibodies were visualized by using Alexa Fluor  
439 488- or 555-conjugated, goat anti-rat or anti-rabbit IgG (H+L, 1:1,000-1:2,000, Life  
440 Technologies, Grand Island, NY, USA). All sections were stained with DAPI (1:5,000, Sigma-  
441 Aldrich, St. Louis, MO, USA). Infected sections stained with secondary Abs and DAPI only  
442 served as negative controls to optimize staining conditions. For each section, at least 6 low- and

443 6 high-magnification fields of the lung sections were imaged on a Carl Zeiss Axio Observer  
444 fluorescence microscope (Carl Zeiss Microscopy LLC, Thornwood, NY, USA) equipped with  
445 ApoTome and Zen imaging software. Acquisition settings were identical among samples of  
446 different experimental groups. Representative images at each time point are presented. To  
447 measure fluorescent intensity of given markers, gray levels across the entire tissue image (low-  
448 magnification, 4 independent images per group) were measured via ImageJ under the same  
449 parameter setting. Data are presented at mean  $\pm$  SEM of the group.

450

451 For *in vitro* studies, cells were seeded onto coverslips in 24-well plates (Falcon Corning,  
452 Corning, NY, USA). At indicated times of infection, slides were washed, fixed with 4% PFA for  
453 20 min, and permeabilized with Triton X-100 for 15 min. After blocking with 10% BSA/3%  
454 goat serum for 1 h, cells were incubated with serum collected from *Orientia*-infected mice  
455 (1:1,500) or rabbit anti-IBA-1 (1:250, Abcam) (1:50) at 4°C overnight and then with a secondary  
456 Ab: goat anti-mouse Alexa Fluor 488 (Thermo Fisher Scientific, Waltham, MA, USA) or donkey  
457 anti-rabbit Alexa Fluor 594 (Invitrogen/Thermo Fisher Scientific) and DAPI (1:1000, Thermo  
458 Fisher Scientific). The cover slips were mounted on slides by using an Antifade Mountant  
459 solution (Invitrogen/Thermo Fisher Scientific). Images were taken using an Olympus IX51  
460 microscope (Olympus Corporation, Tokyo, Japan).

461

#### 462 **Flow cytometry**

463 Equivalent portions of lung tissues were harvested from infected and control mice, minced, and  
464 digested with 0.05% collagenase type IV (Gibco/Thermo Fisher Scientific) in Dulbecco's  
465 Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) for 30 mins at 37°C.

466 Minced tissues were loaded into Medicons and homogenized using a BD Mediamachine System  
467 (BD Biosciences, Franklin Lakes, NJ). Lung single-cell suspensions were made by passing lung  
468 homogenates through 70- $\mu$ m cell strainers. Spleen homogenates were made by passing tissue  
469 through a 70- $\mu$ m strainer. Lymphocytes were enriched by using Lympholyte-M Cell Separation  
470 Media (Burlington, NC). Red blood cells were removed by using Red Cell Lysis Buffer (Sigma-  
471 Aldrich). Leukocytes were stained with the Fixable Viability Dye (eFluor 506)  
472 (eBioscience/Thermo Fisher Scientific, Waltham, MA) for live/dead cell staining, blocked with  
473 Fc $\gamma$ R blocker, and stained with fluorochrome-labeled antibodies (Abs). The following Abs  
474 purchased from Thermo Fisher Scientific and BioLegend (San Diego CA): PE-Cy7-anti-CD3 $\epsilon$   
475 (145-2C11), Pacific Blue-anti-CD4 (GK1.5), APC-Cy7-anti-CD8a (53-6.7), APC-anti-Ly6G  
476 (1A8-Ly6G), APC-anti-CD31 (390), PE-anti-VEGFR2 (Avas12a1), FITC-anti-ICAM-1  
477 (YN1/1.7.4), Pacific Blue-anti-CD45 (30-F11), PE-anti-CD80 (16-10A1), BV421-anti-CD206  
478 (CO68C2), FITC-anti-CD64 (X54-5/7.1), PerCP-Cy5.5-anti-CD11b (M1/70). Cells were fixed in  
479 2% paraformaldehyde overnight at 4°C before cell analysis. Data were collected by a BD  
480 LSRFortessa (Becton Dickinson, San Jose, CA) and analyzed using FlowJo software version  
481 8.86 (Tree Star, Ashland, OR). As previously reported for mouse lung tissues [65] CD45<sup>+</sup>CD31<sup>-</sup>  
482 and CD45<sup>-</sup>CD31<sup>+</sup> cells were considered hematopoietic cells and endothelial cells by flow  
483 cytometry, respectively.

484

#### 485 **Western blot**

486 Protein from lung tissues was extracted with a RIPA lysis buffer (Cell Signaling Technology,  
487 Danvers, MA) and quantified with BCA Protein Assay kit (Thermo Fisher Scientific). Protein  
488 samples (40  $\mu$ g/lane) were loaded onto 4-20% SDS-PAGE gels (Bio-Rad Laboratories, Hercules,

489 CA, USA) and transferred onto nitrocellulose membranes (Bio-Rad Laboratories). After  
490 blocking non-specific binding sites, membranes were respectively incubated with rabbit Abs  
491 specific to mouse Tie2 (1:500, Abcam), phospho-Tie2 (1:400, R&D System, USA), and  $\beta$ -actin  
492 (1:15000, Novus Biologicals, USA), and an anti-rabbit secondary antibody (SouthernBiotech,  
493 Birmingham, AL, USA). After treatment with the Maximum Sensitivity Substrate (Thermo  
494 Fisher Scientific) for 1 min, the light signals were captured by Luminescent Image Analyzer  
495 (ImageQuant LAS 4000, GE Healthcare Bio-Sciences AB, Sweden). Protein bands were  
496 quantified by using image analysis software (ImageJ). Three independent experiments were  
497 performed.

498

499

#### 500 **Infection of mouse bone marrow-derived M $\Phi$ s**

501 Bone marrow cells were collected from mouse femur and tibia and treated with a red cell lysis  
502 buffer (Sigma). For M $\Phi$  generation, bone marrow cells were grown in DMEM (Gibco) with 10%  
503 FBS, penicillin/streptomycin antibiotics, and 40 ng/ml M-CSF (BioLegend) and incubated at  
504 37°C. Media was changed at day 3, and cells were collected at day 7 and seeded onto 6- or 24-  
505 well plates for overnight. M $\Phi$ s were treated with either 100 ng/ml LPS (for M1 polarization) or  
506 10 ng/ml mouse rIL-4 (for M2 polarization, Peprotech, Rocky Hill, NJ) for 24 h. Cells were then  
507 infected with *O. tsutsugamushi* (5 MOI) and centrifuged at 2,000 RPM for 5 min to synchronize  
508 infection of the cells.

509



## 510 **Quantitative PCR and reverse transcriptase PCR (qPCR and qRT-PCR)**

511 To determine bacterial loads, bone marrow-derived MΦs were collected at 3, 24, 48, and 72 hpi  
512 by using a DNeasy kit (Qiagen) and used for qPCR assays, as previously described [32].  
513 Bacterial loads were normalized to total nanogram (ng) of DNA per  $\mu\text{L}$  for the same sample, and  
514 data are expressed as the gene copy number of 47-kDa protein per picogram (pg) of DNA. The  
515 copy number for the 47-kDa gene was determined by known concentrations of a control plasmid  
516 containing single-copy insert of the gene. Gene copy numbers were determined via serial dilution  
517 (10-fold) of the control plasmid.

518

519 To measure host gene expression, mouse tissues or *in vitro*-infected cells were respectively  
520 collected in RNALater (Ambion, Austin, TX) or Trizol solution at 4°C overnight to inactivate  
521 infectious bacteria and stored at -80°C for subsequent analyses. Total RNA was extracted by  
522 using RNeasy mini kits (Qiagen) and digested with RNase-free DNase (Qiagen); cDNA was  
523 synthesized with the iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA). The  
524 quantitative RT-PCR (qRT-PCR) assays were performed with iTaq SYBR Green Supermix and a  
525 CFX96 Touch Real-Time PCR Detection System (Bio-Rad). PCR assays were denatured for 3  
526 min at 95°C, followed by 40 cycles of 10s at 95°C and 30s at 60°C. Melt-curve analysis was also  
527 used to check the specificity of the amplification reaction. Relative abundance of transcripts was  
528 calculated by using the  $2^{-\Delta\Delta\text{CT}}$  method and compared to housekeeping genes glyceraldehyde-3-  
529 phosphate dehydrogenase (GAPDH) or  $\beta$ -actin. Primers used in these analyses are listed in Table  
530 S1.

531

## 532 **Human umbilical vein endothelial cell (HUVEC) infection**

533 HUVECs (Cell Application, San Diego, CA) were maintained in complete Prigrow I medium  
534 supplemented with 3% heat-inactivated FBS (Applied Biological Materials, Vancouver, Canada)  
535 in 5% CO<sub>2</sub> at 37°C. All *in vitro* experiments were performed between cell passages 5 and 7, as  
536 described previously [33, 66]. For infection, HUVECs were cultivated in Prigrow I medium with  
537 10% FBS and seeded onto 6-well plates (Corning Inc., Corning, NY). Confluent monolayers  
538 were infected with *Orientia* (3 and 10 MOI) for 24 h and compared with uninfected controls.

539

#### 540 **Human serum collection and measurement of Ang2 by ELISA**

541 Human serum samples were collected from healthy volunteers ( $n = 8$ ) and scrub typhus patients  
542 ( $n = 32$ ) after obtaining informed consent at the Chungnam National University Hospital in  
543 Daejeon, South Korea. Scrub typhus diagnosis was confirmed based on clinical symptoms and a  
544 positive serology: a 4-fold or greater rise in the titer of paired plasma or single cut-off titer of an  
545 IgM antibody  $\geq 1:160$  by an indirect immunofluorescence antibody assay (IFA) against *O.*  
546 *tsutsugamushi* antigens or passive hemagglutination assay (PHA) during hospital admission.  
547 Healthy volunteers had never been previously diagnosed with scrub typhus, and their sera were  
548 negative when examined by IFA. Patient plasma samples were classified into four groups based  
549 on their IFA titers. Ang2 concentration was determined by using a commercial ELISA kit  
550 (Abcam), according to manufacturer's instructions.

551

#### 552 **Human antibody titer measured by IFA**

553 L929 cells infected with three strains of *O. tsutsugamushi* (Boryong, Karp, and Gilliam strains)  
554 were harvested, mixed in equal amounts, and used as antigens to measure total IgG titers against  
555 *O. tsutsugamushi* via IFA. Briefly, infected L929 cells were harvested, washed with PBS, seeded

556 onto Teflon-coated spot slides, and fixed with cold acetone for 10 min. The slides were stored at  
557  $-70^{\circ}\text{C}$  until use. Two-fold serially diluted (1:40 to 1:1280 in PBS) patient sera was added to the  
558 antigen-coated spot on the slide and incubated for 30 min in a wet chamber at room temperature.  
559 An Alexa Fluor 488-conjugated goat anti-human IgG (diluted 1:1000 in PBS, Molecular Probes,  
560 Waltham, MA, USA) was used as the secondary antibody. The stained slides were examined  
561 under an Olympus FV1000 laser scanning confocal microscope (Olympus, Tokyo, Japan). The  
562 endpoint titer of IFA was defined as the highest titer showing a fluorescence signal above the  
563 background.

564

### 565 **Statistical analysis**

566 Data were presented as mean  $\pm$  standard errors of the mean (SEM). Differences between  
567 individual treatment and control groups were determined by using Student's t test, utilizing  
568 Welch's correction when appropriate. One-way ANOVA was used for multiple group  
569 comparisons with a Tukey's Post Hoc for comparisons between groups. Statistically significant  
570 values are referred to as \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$ ; and \*\*\*\*,  $p < 0.0001$ .

571

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577

578

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## 853 **Figure Legends**

854

### 855 **Fig 1. Endothelial cell (EC) activation and vascular damage in the lungs of *O.***

856 ***tsutsugamushi*-infected mice and in infected HUVECs *in vitro*.** Female C57BL/6J mice were

857 inoculated with  $1.325 \times 10^6$  of *O. tsutsugamushi* Karp strain (4-5 mice/group) or PBS (3-4

858 mice/group). At days 2, 6, or 9 post-infection, equivalent lung portions were collected for

859 analyses. (A) Frozen lung sections were co-stained for *Orientia* bacteria (red), ICAM-1 (green),

860 and DAPI (blue, top row, scale bar = 50  $\mu$ m) with close-up views of the boxed areas in the

861 bottom row (bar = 20  $\mu$ m). (B) Lung sections were co-stained for VE-cadherin (adherens

862 junctions, red), FITC-labeled I-B<sub>4</sub> lectins (green), and DAPI (blue, top row, bars = 50  $\mu$ m).

863 Close-up views of the boxed areas located the bottom row (bar = 20  $\mu$ m). (C) Quantification of

864 fluorescent ICAM-1 and VE-Cadherin staining (four images per time point). (D) Lung-derived

865 cells were analyzed via flow cytometry for the percentage of ICAM-1<sup>+</sup> and VEGFR2<sup>+</sup> cells

866 among gated CD31<sup>+</sup>CD45<sup>-</sup> ECs (4-5 mice/group in infected groups; 3 mice/group in PBS

867 groups). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$  compared to PBS controls.

868 Graphs are shown as mean +/- SEM. Flow cytometric and qRT-PCR data were analyzed by

869 using one-way ANOVA with Tukey's Post Hoc. At least 4 independent mouse infection

870 experiments and 2 independent *in vitro* experiments were performed with similar trends, and

871 shown are representative data.

872

### 873 **Fig 2. Elevated Ang2 expression and decreased Ang1 expression during *O. tsutsugamushi***

874 **infection.** Mice were infected as in *Fig. 1*. (A) Frozen lung sections were co-stained for Ang1 (a

875 marker for endothelial quiescence, green), Ang2 (an endothelial stress marker, red), and DAPI



876 (blue) showing images at a low magnification (top row, scale bar = 50  $\mu\text{m}$ ) and close-up views  
877 of the boxed areas (bottom row, bar = 20  $\mu\text{m}$ ). (B) Quantification of fluorescent Ang1 and Ang2  
878 staining (four images per time point). (C) Human serum Ang2 proteins in the control subjects  
879 (CNT) or scrub typhus patients (8/group) with different anti-*Orientia* IFA antibody titers were  
880 measured by ELISA. Shown are data from two independent experiments. \*,  $p < 0.05$ ; \*\*,  $p <$   
881 0.01 and \*\*\*,  $p < 0.001$ ; \*\*\*\*,  $p < 0.0001$  compared to the controls. Graphs are shown as mean +/-  
882 SEM. Serum ELISA and qRT-PCR groups were analyzed via one-way ANOVA with Tukey's  
883 Post Hoc. At least 3 independent mouse infection experiments were performed with similar  
884 trends, and shown are representative data.

885 **Fig 3. Reduced Tie2 expression and activation in the lungs of *O. tsutsugamushi* infected**  
886 **mice.** Frozen lung tissue sections were stained for the Tie2 receptor (red) and DAPI (blue, bar =  
887 50  $\mu\text{m}$ ). (B) Lung tissue homogenates (40  $\mu\text{g}/\text{lane}$ ) were measured by Western blots for the  
888 levels of phospho-Tie2 (pTie2) and total Tie2 proteins and compared with the GAPDH controls.  
889 (C) *TIE2* mRNA levels in mouse lungs were measured via qRT-PCR; data are presented as  
890 relative mRNA values normalized to GAPDH. \*\*,  $p < 0.01$  compared to the controls. Graph  
891 shown as mean +/- SEM. Serum ELISA and qRT-PCR groups were analyzed via one-way  
892 ANOVA with Tukey's Post Hoc.

893 **Fig 4. Polarized M $\Phi$  activation in infected mouse lungs.** Mice were infected with *O.*  
894 *tsutsugamushi* (4-5 mice/group) or PBS (3-4 mice/group) for lung tissues collection at indicated  
895 days of infection, as in *Fig. 1*. (A) Flow cytometric analyses of lung-derived cells, gated on  
896 CD11b<sup>+</sup>Ly6G<sup>-</sup> M $\Phi$ s and M $\Phi$  subsets, are shown for the D9 samples. The percentages and total  
897 numbers of (B) activated M $\Phi$ s (CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>), (C) M1-type M $\Phi$ s

898 (CD80<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>), as well as (D) M2-type MΦs (CD206<sup>+</sup>CD64<sup>+</sup>CD11b<sup>+</sup>Ly6G<sup>-</sup>) are  
899 shown, respectively.

900 **Fig 5. Transcription of M1 and M2 associated genes in the lung of infected mice.** Lung  
901 tissues were measured for the expression of M1-related genes (A) and M2-related genes (B),  
902 respectively. Data are presented as relative to β-actin values. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p <$   
903  $0.001$  compared to the PBS controls. Graphs are shown as mean +/- SEM. One-way ANOVA  
904 with Tukey's Post Hoc was used for statistical analysis. Two independent mouse infection  
905 experiments were performed with similar trends, and shown are representative data.

906

907 **Fig 6. Enhanced bacterial growth in M2-polarized MΦs.** Bone marrow-derived MΦs were  
908 generated from C57BL/6J mice, polarized into M1 or M2 types by pre-treatment of cells with  
909 LPS (100 ng/ml) or rIL-4 (10 ng/ml), and infected with bacteria (5 MOI). (A) Bacterial loads at  
910 3, 48, and 72 hpi (n = 5) were determined by qPCR. Data are presented as the *Orientia* 47-kDa  
911 gene copy per pg of DNA. (B) Cells were co-stained for *Orientia* (green), IBA-1 (a MΦ marker,  
912 red), and DAPI (blue) at 3 and 72 hpi. \*,  $p < 0.05$ . Data for qPCR were analyzed via a Mann-  
913 Whitney t test. Three independent experiments were performed with similar trends, and shown  
914 are representative data.

915

## 916 **Supporting information**

917 **S1 Fig. Pulmonary pathology progression and reduced expression of occludin tight**  
918 **junction proteins in infected lung tissues.** Female C57BL/6J mice (4-6 mice/group) were

919 inoculated with  $1.325 \times 10^6$  of *O. tsutsugamushi* Karp strain. At indicated days of infection,  
920 equivalent lung portions were collected. (A) Hematoxylin and eosin staining of lung tissues  
921 during lethal challenge demonstrating increased cellular infiltration and alveolar thickening as  
922 the infection progresses (scale bars = 50  $\mu\text{m}$ ). (B) Frozen sections were processed for  
923 immunofluorescent staining and co-stained for occludin (cell-cell tight junctions, red), FITC-  
924 labeled GSL I-B<sub>4</sub> lectins (green, top rows, scale bars = 50  $\mu\text{m}$ ), and DAPI (blue). The close-up  
925 views of the boxed areas are shown in the lower row (bar = 20  $\mu\text{m}$ ). (C) Flow cytometry analysis  
926 of viable pulmonary ECs ( $\text{CD31}^+\text{CD45}^-$ ) collected at early (D0) and late (D9) infection. (D)  
927 Cultured HUVECs were infected with bacteria at 3 or 10 multiplicity of infection (MOI, 4  
928 samples/group) and analyzed via qRT-PCR for gene expression at 24 h post-infection. Data are  
929 presented as relative to GAPDH values. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*,  $p < 0.001$  compared  
930 to PBS controls. Graphs are shown as mean  $\pm$  SEM. Flow cytometric and qRT-PCR data were  
931 analyzed by using one-way ANOVA with Tukey's Post Hoc. At least 3 independent mouse  
932 infection experiments and 2 independent *in vitro* experiments were performed with similar  
933 trends, and shown are representative data.

934

935 **S2 Fig. Leukocyte and T cell recruitment in the lungs of lethally challenged mice.** Female  
936 C57BL/6J mice (3-5 mice per group) were inoculated with  $1.325 \times 10^6$  of *O. tsutsugamushi* Karp  
937 strain. At indicated days of infection, equivalent lung portions were collected and processed for  
938 immunofluorescent staining or flow cytometric analysis. (A) Frozen sections were either co-  
939 stained for Ang2 (red) and CD45 (a leukocyte marker, green), or Ang2 (green) and CD3 (a T cell  
940 marker, red, bars = 50  $\mu\text{m}$ ). The percentage and absolute number of  $\text{CD3}^+\text{CD4}^+$  T cells (B), as  
941 well as  $\text{CD3}^+\text{CD8}^+$  T cells (C), were quantified and compared to non-infected controls

942 (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*\*,  $p < 0.0001$ ). Graphs are shown as +/- SEM. Flow cytometry groups  
943 were analyzed using one-way ANOVA with Tukey's Post Hoc.

944

945 **S3 Fig. MΦ infection in mouse lungs and differentiation *in vitro*.** (A) Female C57BL/6J mice  
946 (4-6 mice per group) were inoculated with  $1.325 \times 10^6$  of *O. tsutsugamushi* Karp strain. At days  
947 2 and 10, equivalent lung portions were processed; frozen sections were co-stained for *Orientia*  
948 (red), IBA-1 (green, a macrophage marker), and DAPI (blue), showing images in a low-  
949 magnification (top rows, scale bar = 50 μm) and close-up views of the boxed areas (bottom rows,  
950 bar = 20 μm). (B) Bone marrow-derived MΦs were treated with LPS (100 ng/ml) or rIL-4 (10  
951 ng/ml) for 24 h and analyzed for the expression of indicated markers via flow cytometry. The  
952 numbers represent the percentages (%) of gated cells. (C) LPS- and IL-4-primed cells were  
953 analyzed by qRT-PCR for the expression of the indicated markers, showing the polarization of  
954 MΦ subsets compared with control cells (\*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; and \*\*\*\*,  $p < 0.0001$ ). Data  
955 are shown as +/- SEM and were analyzed using one-way ANOVA with Tukey's Post Hoc.

956 **S1 Table. Real-time PCR primers of human, murine, and bacterial genes.** The primer  
957 sequences used in this study (listed in the 5' to 3' direction).

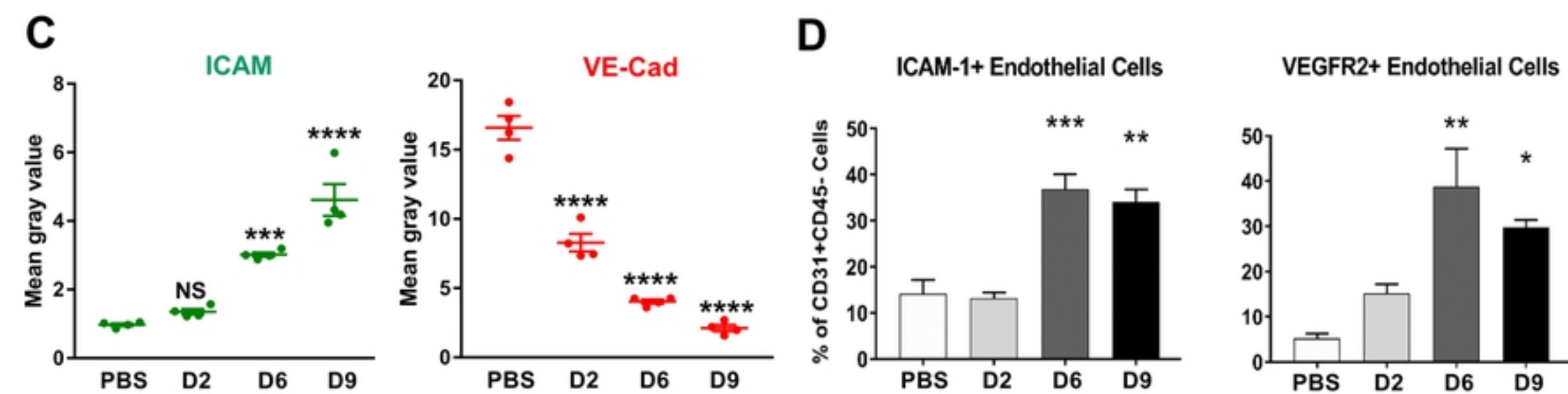
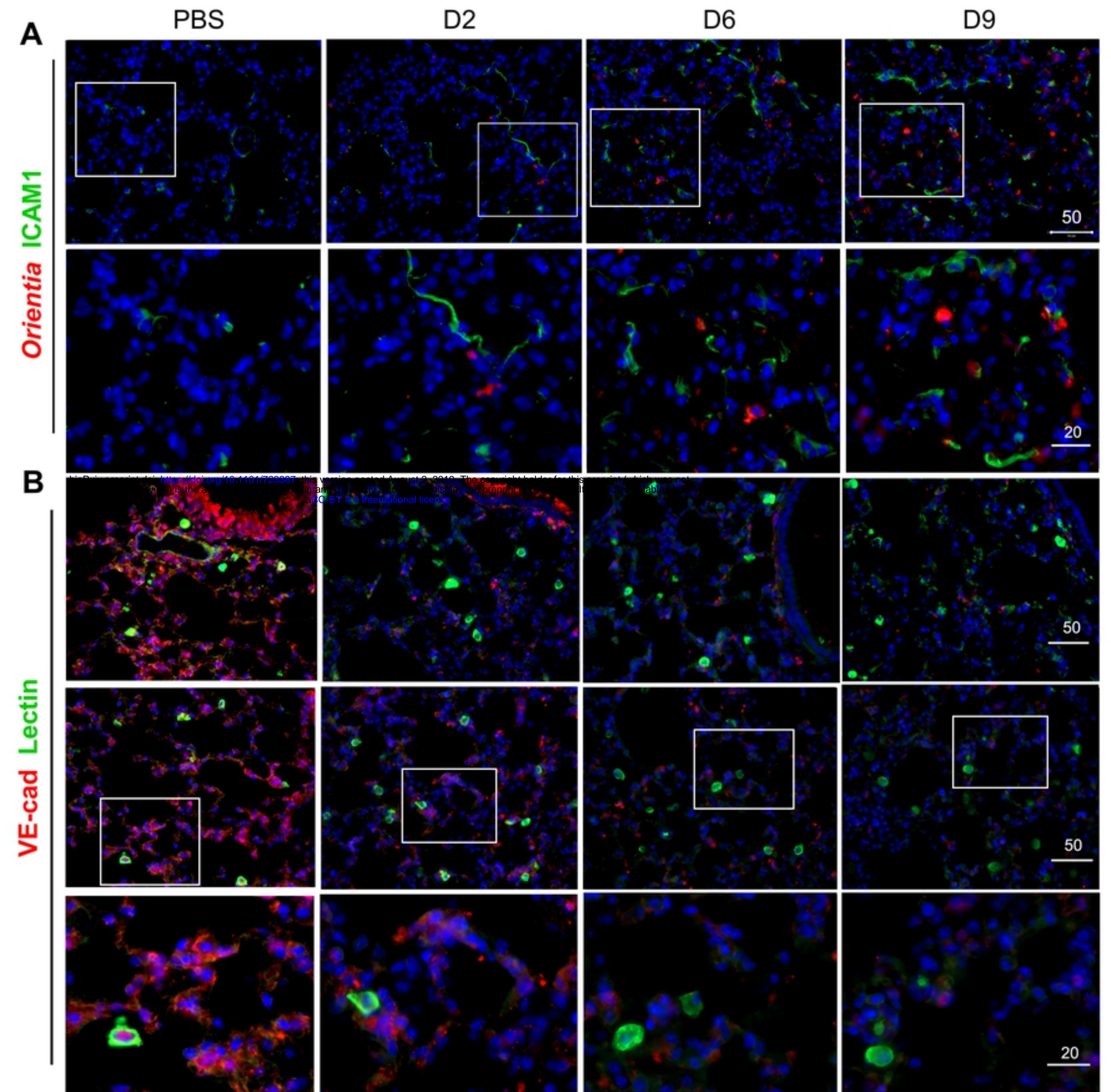


Figure 1

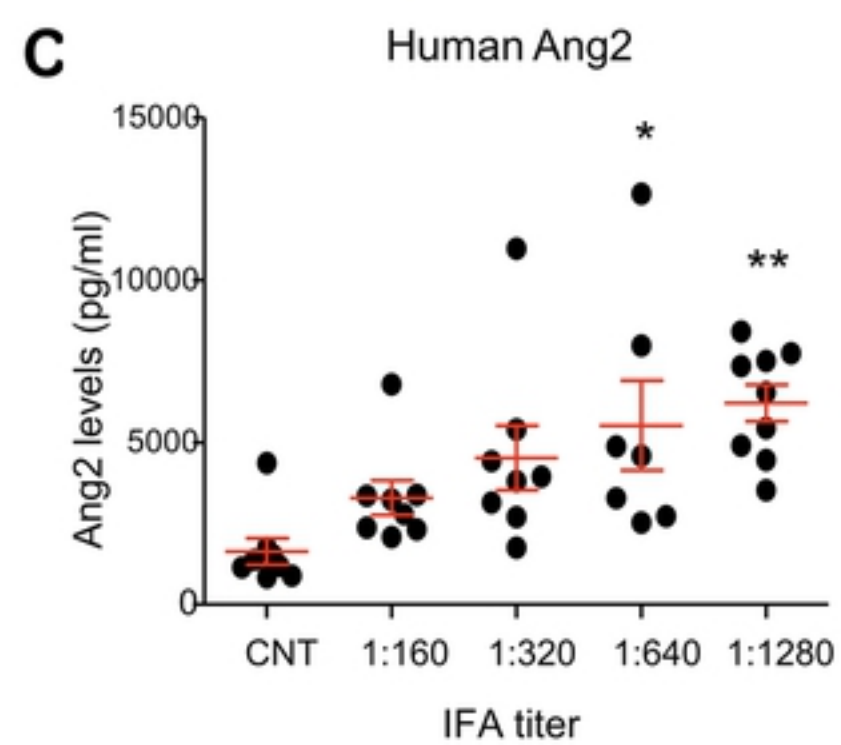
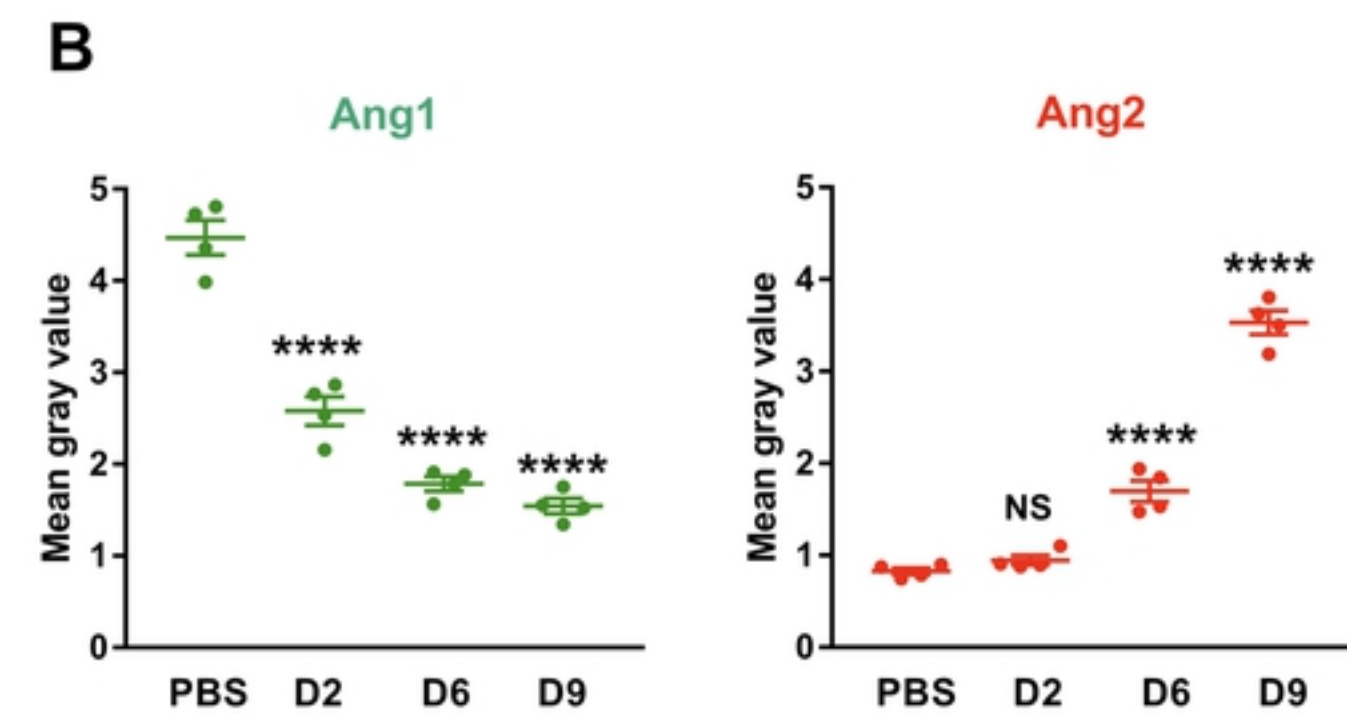
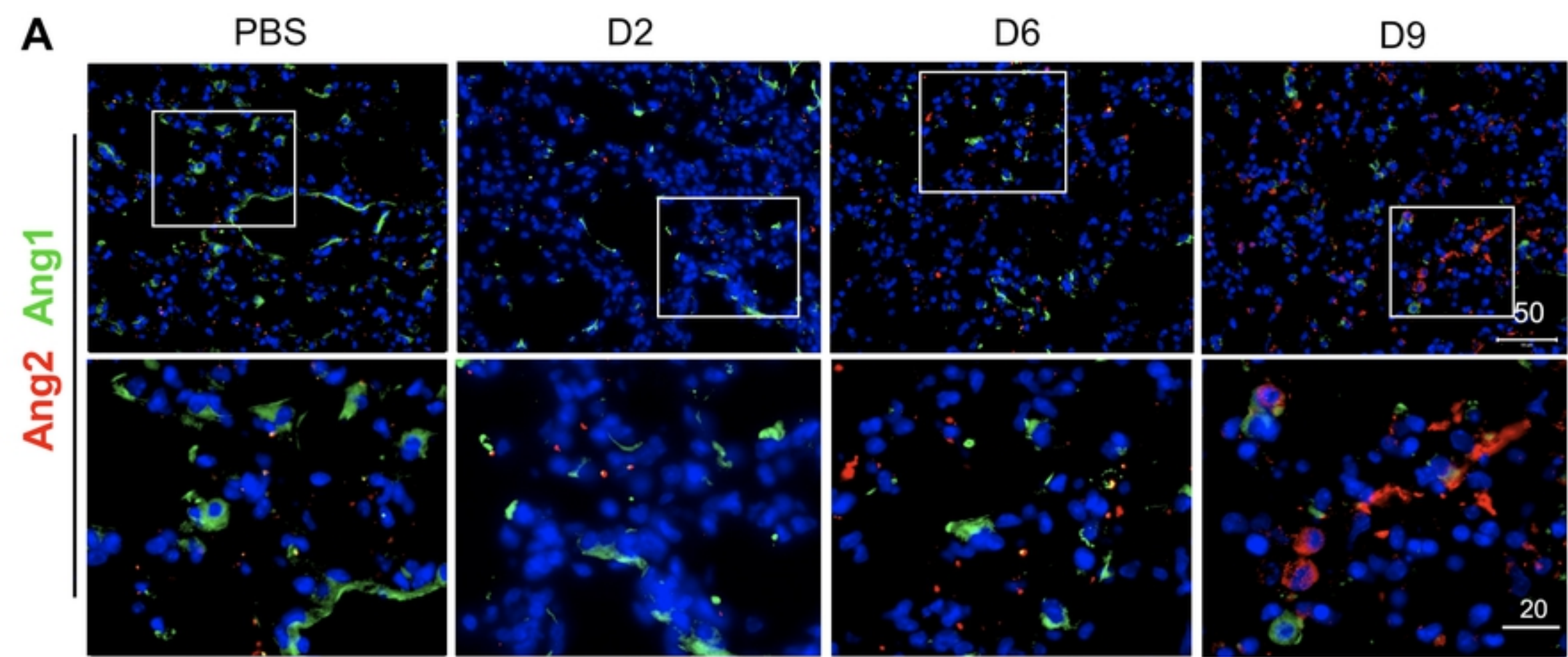


Figure 2

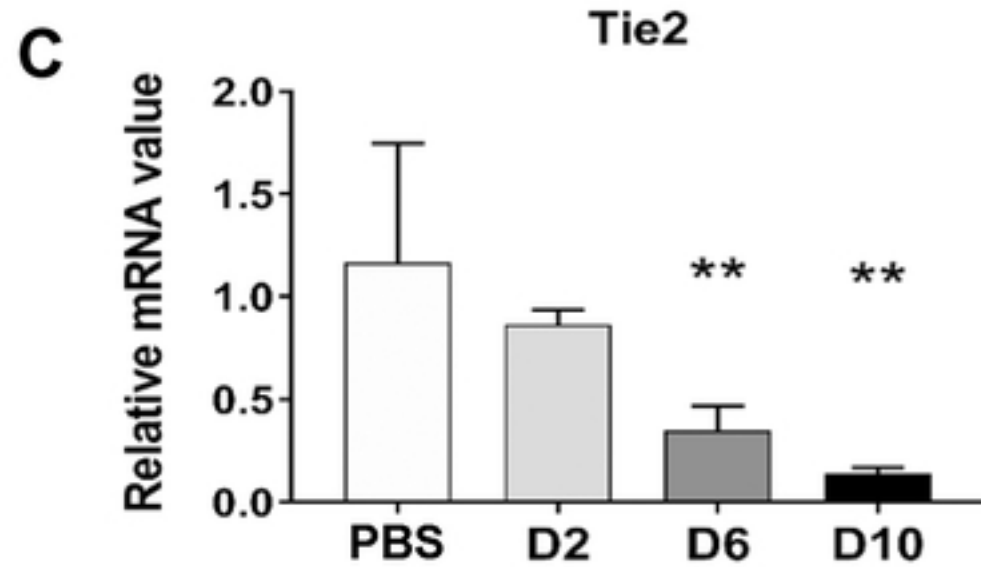
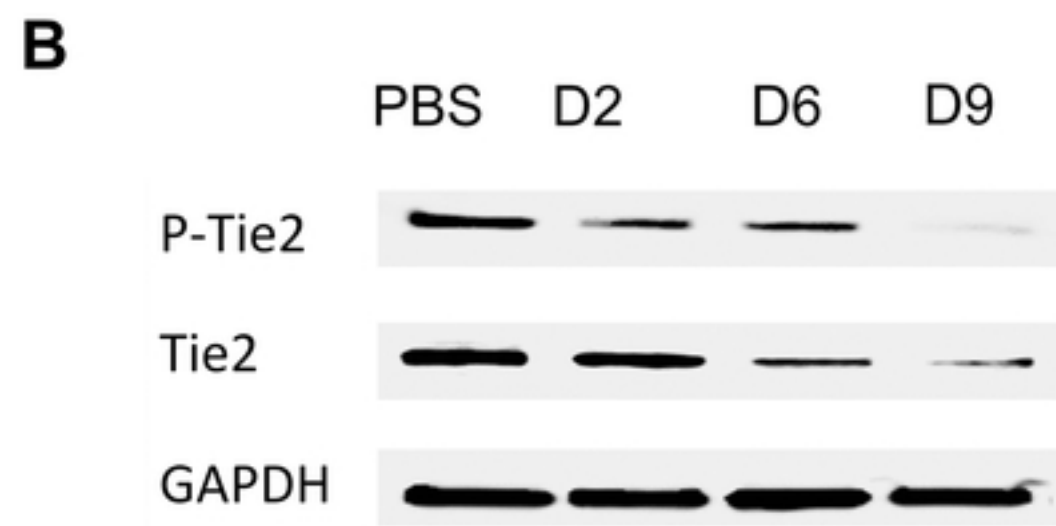
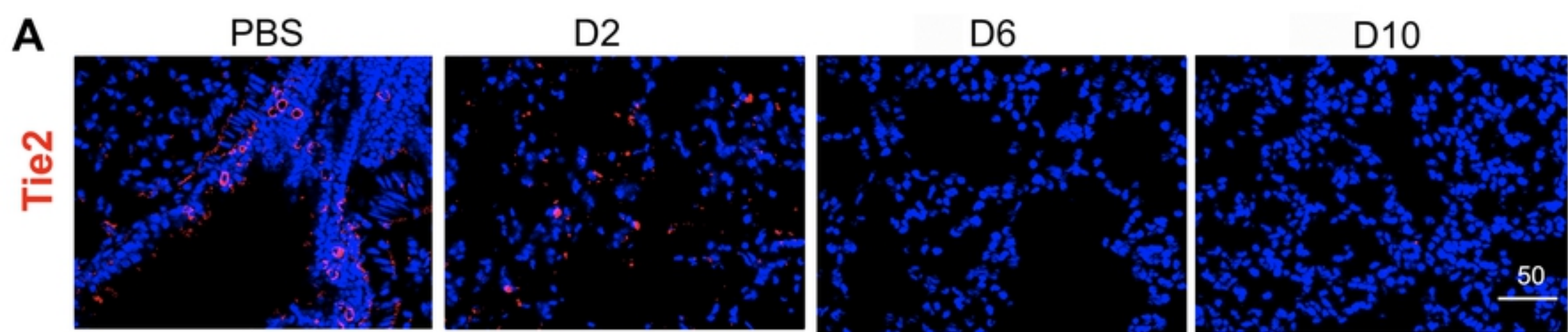


Figure 3

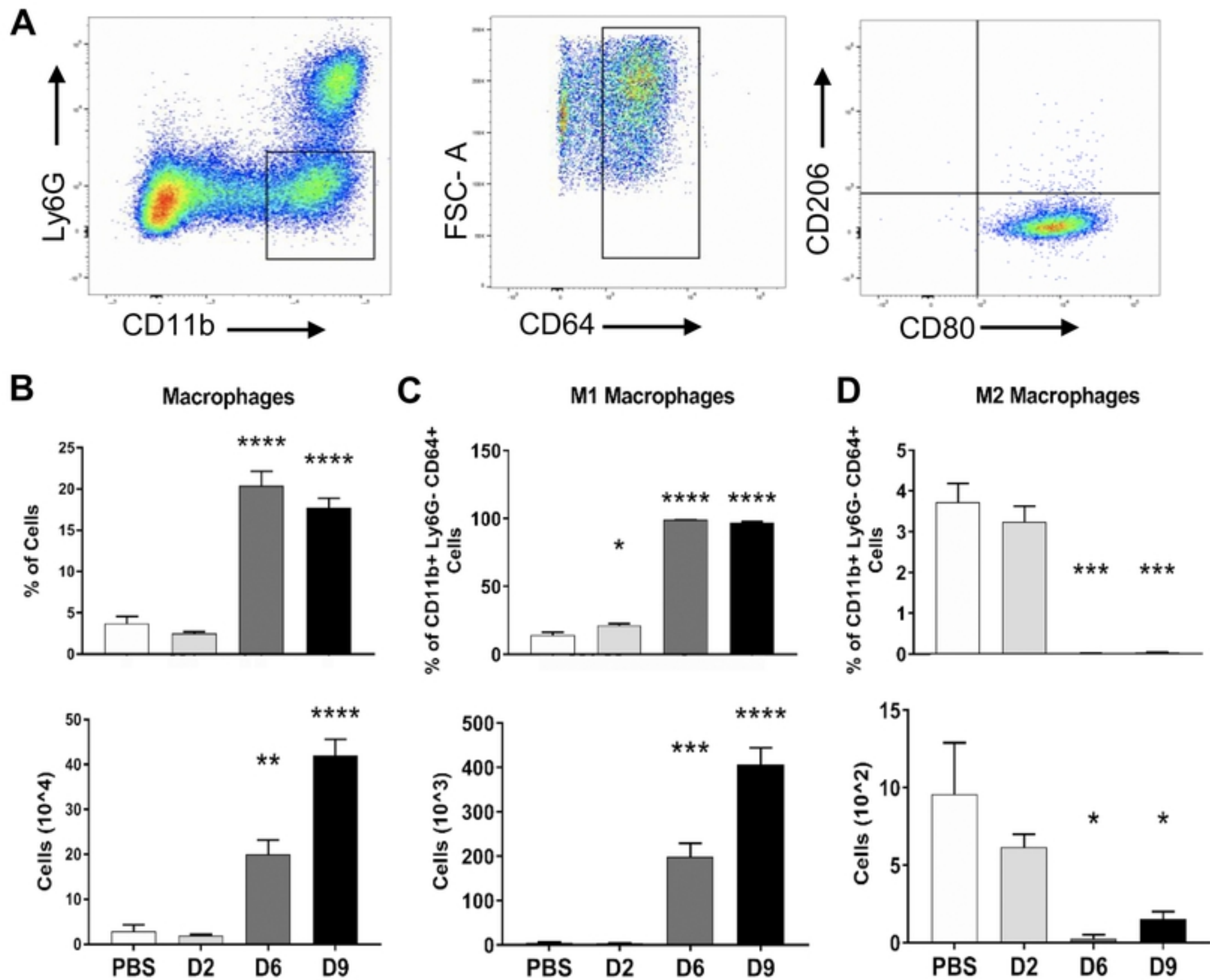


Figure 4



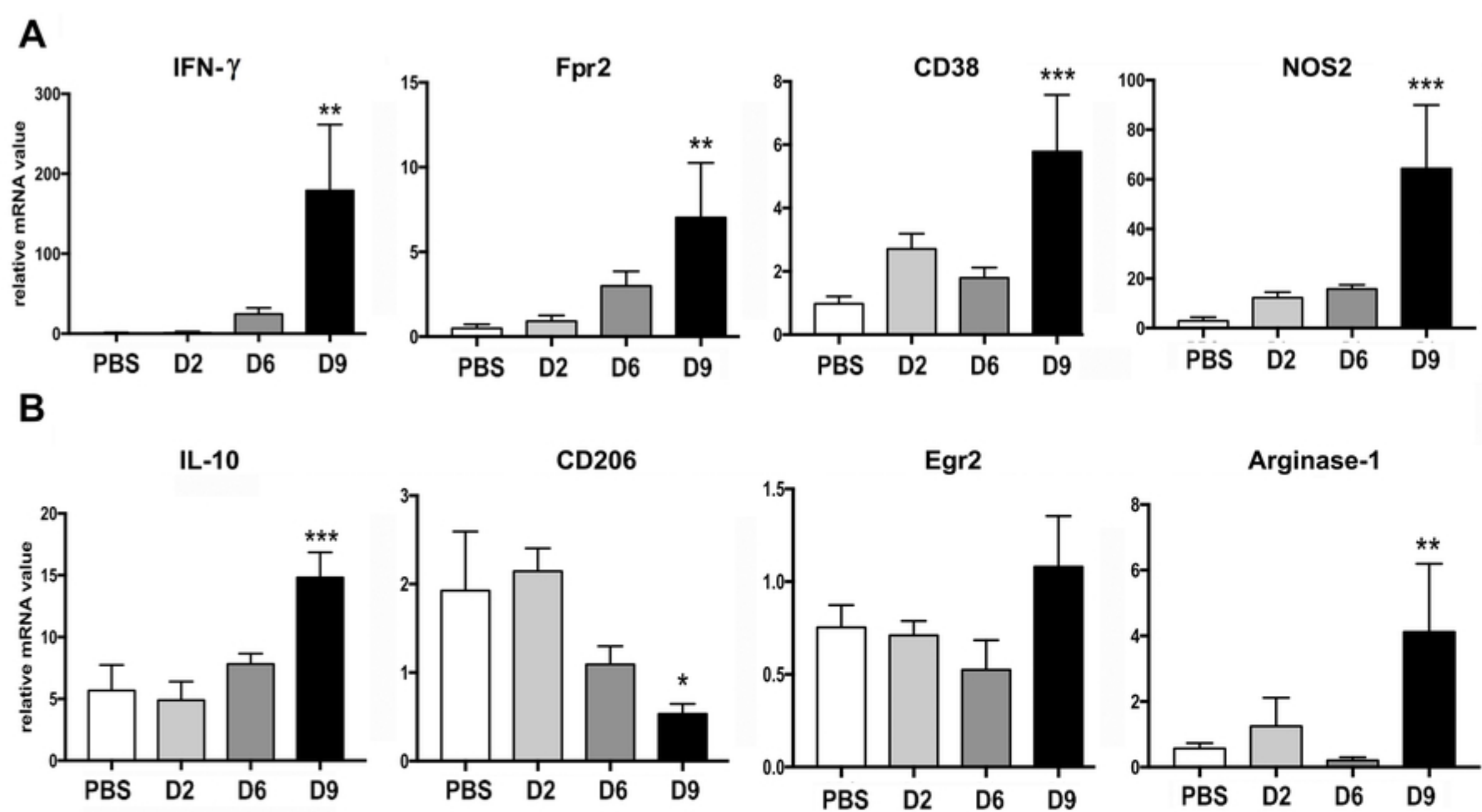


Figure 5

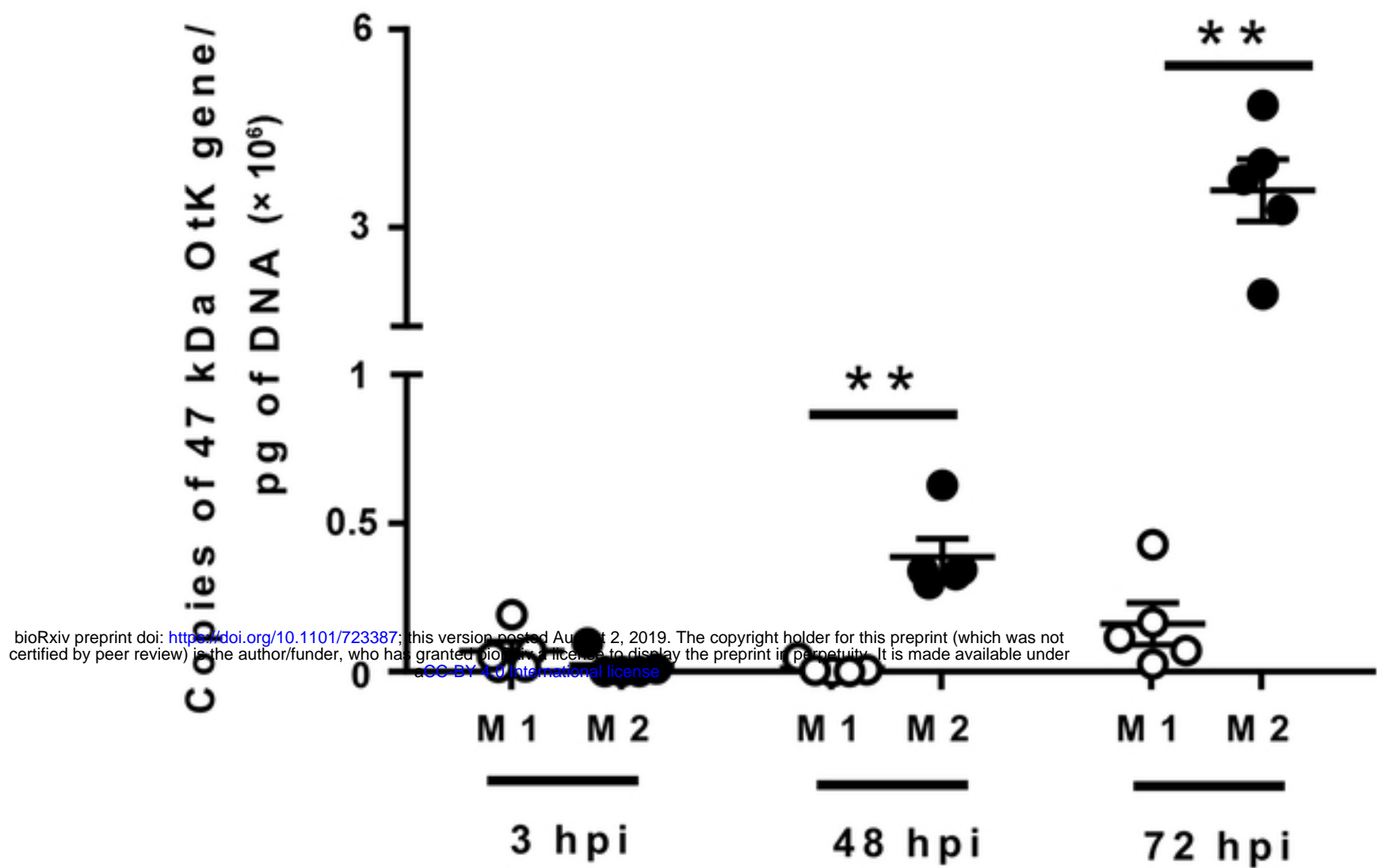
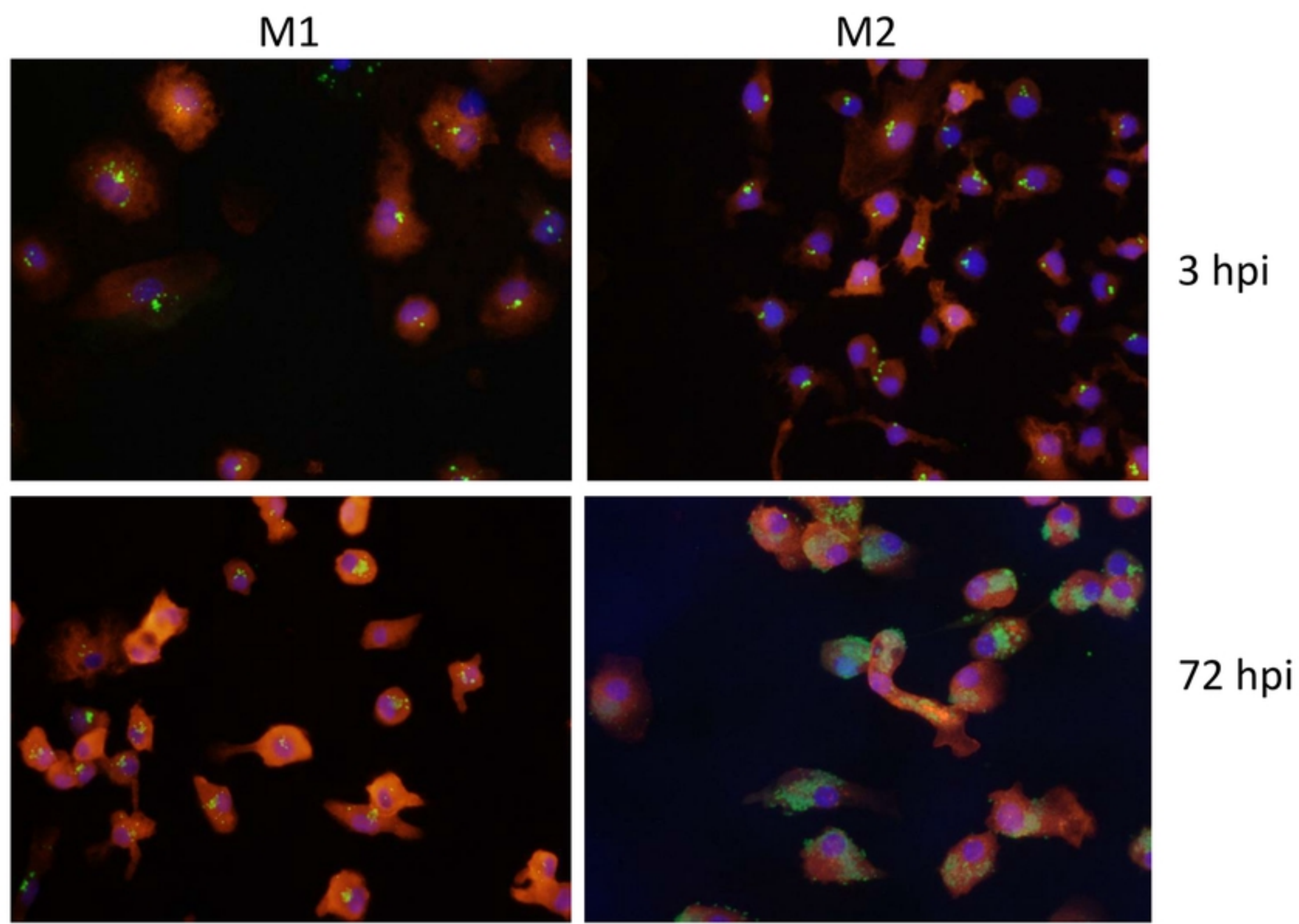
**A****B**

Figure 6

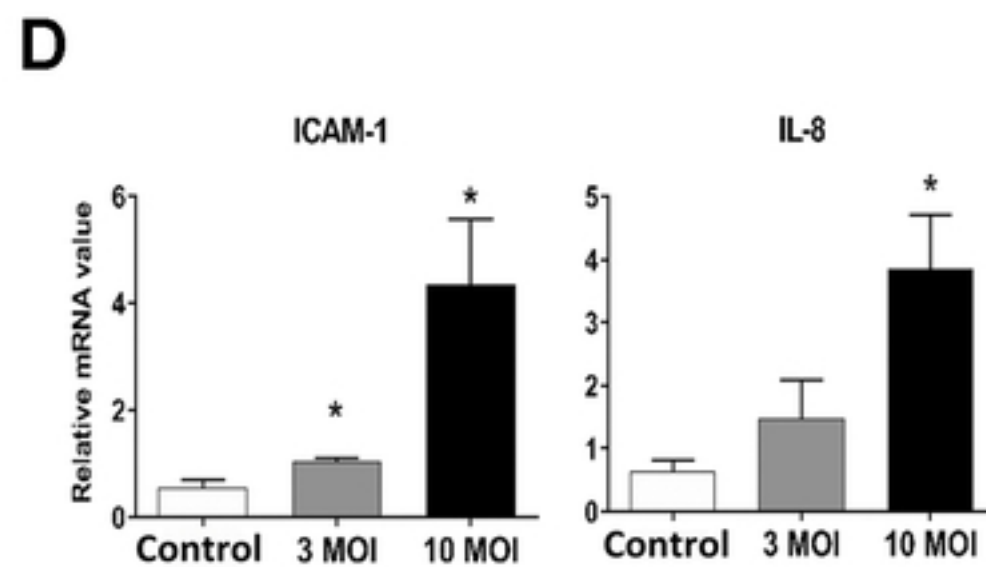
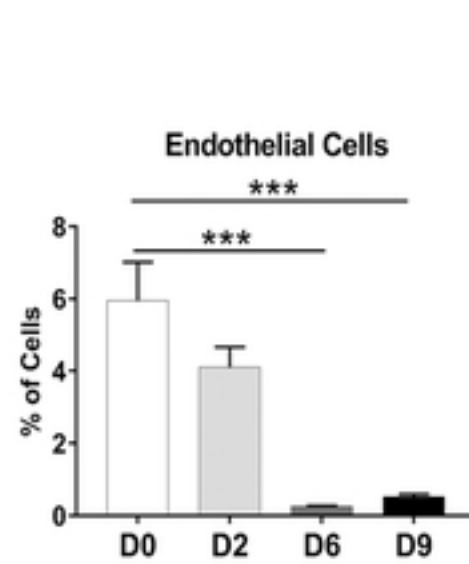
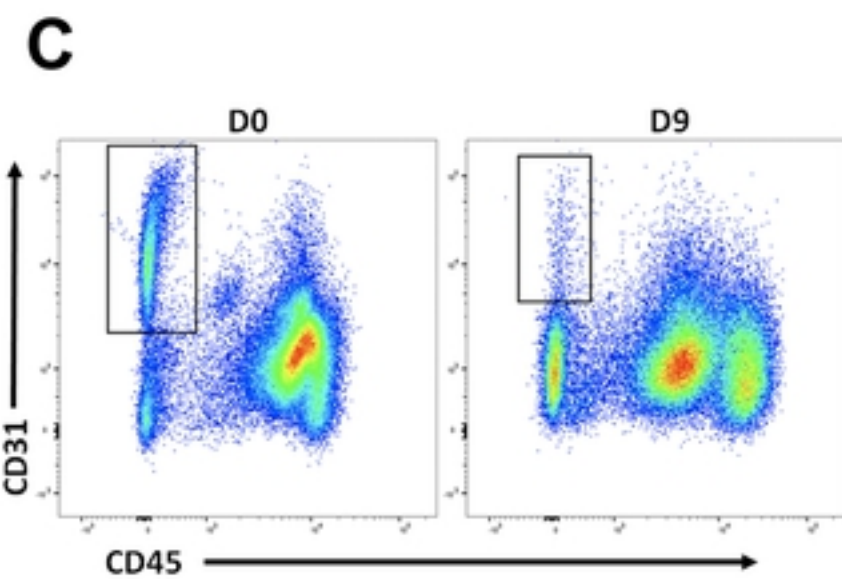
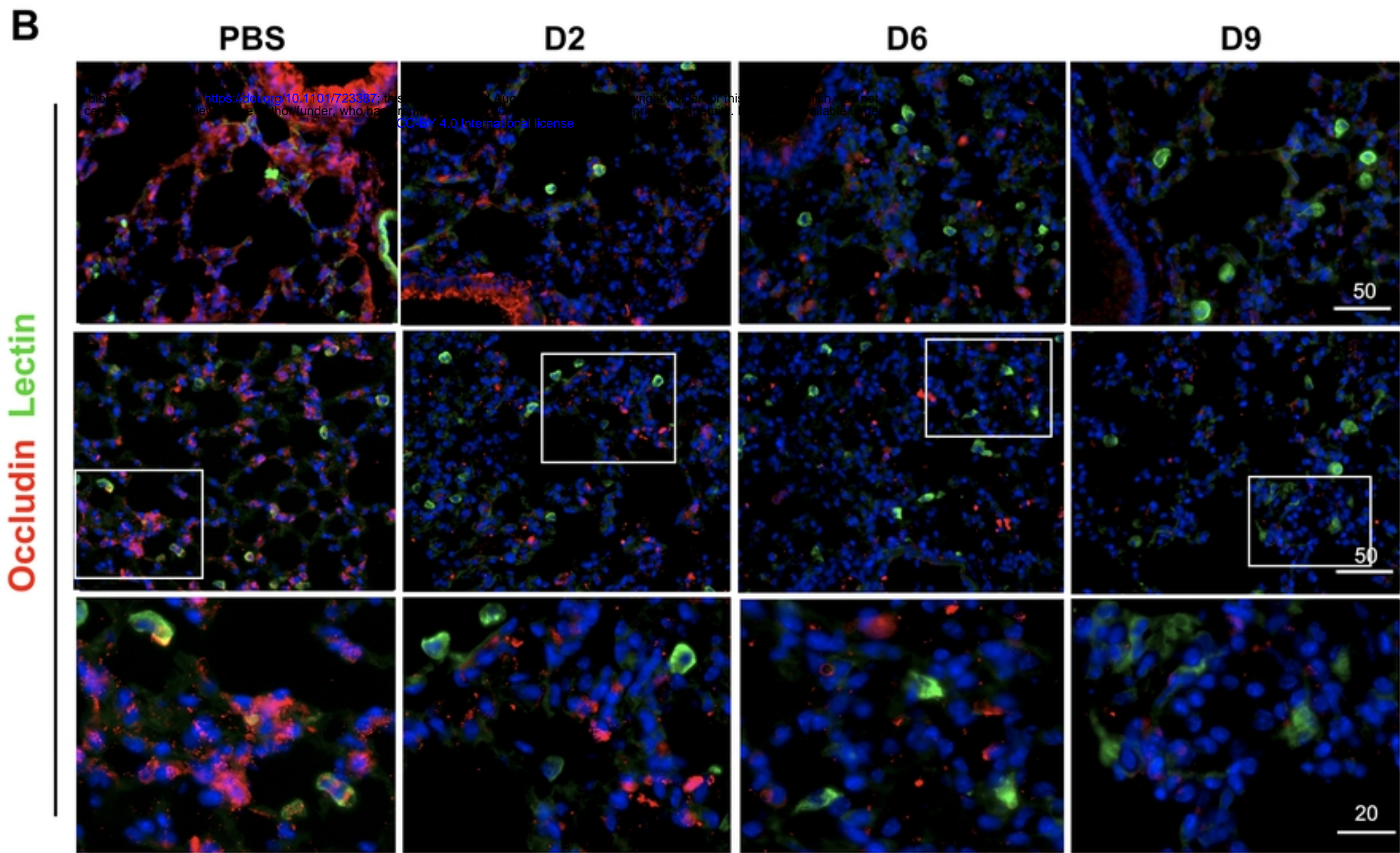
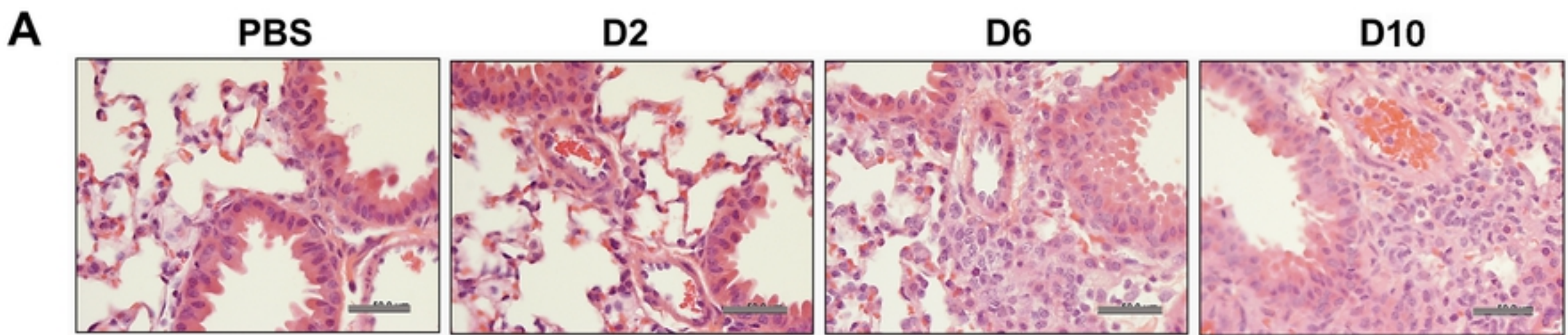


Figure S1

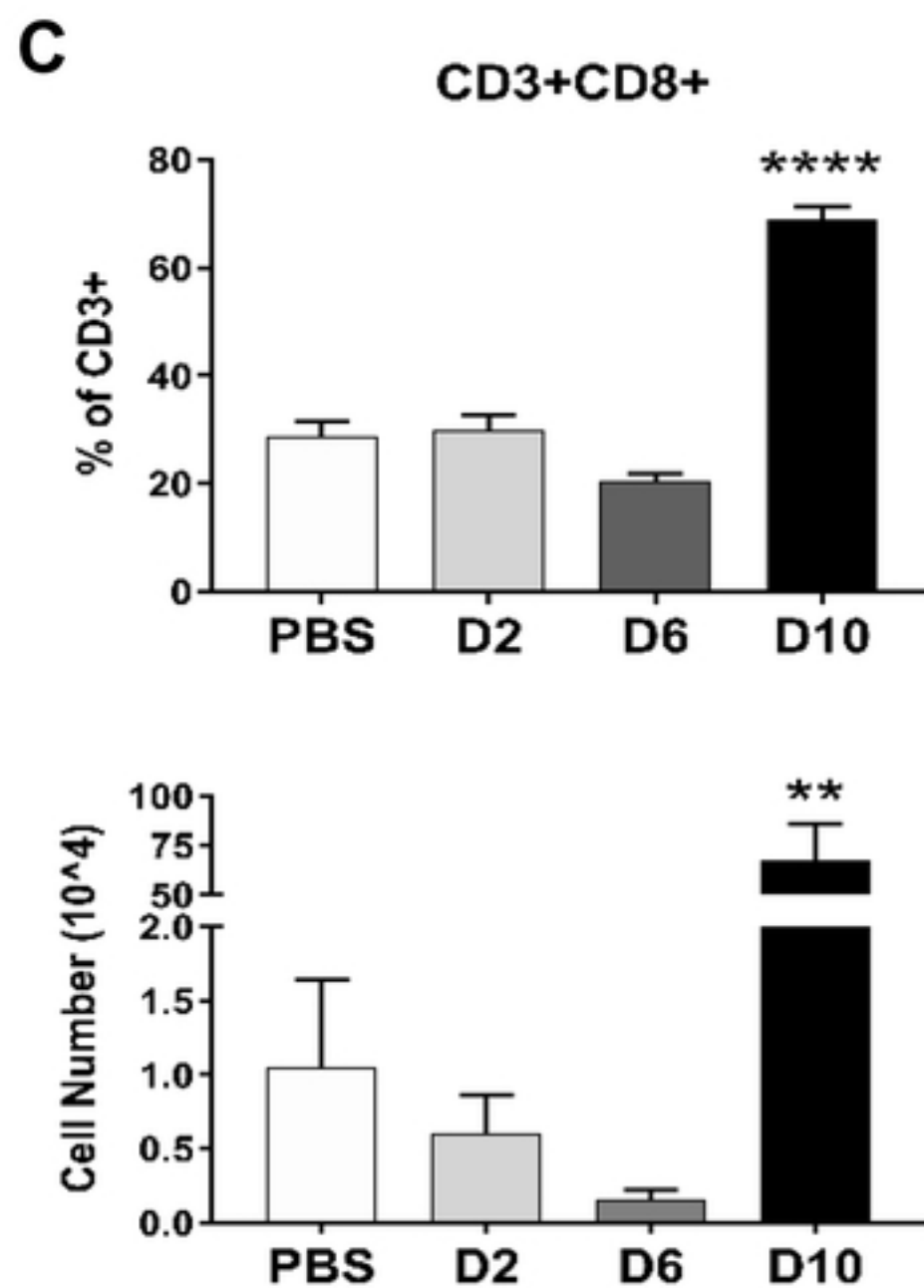
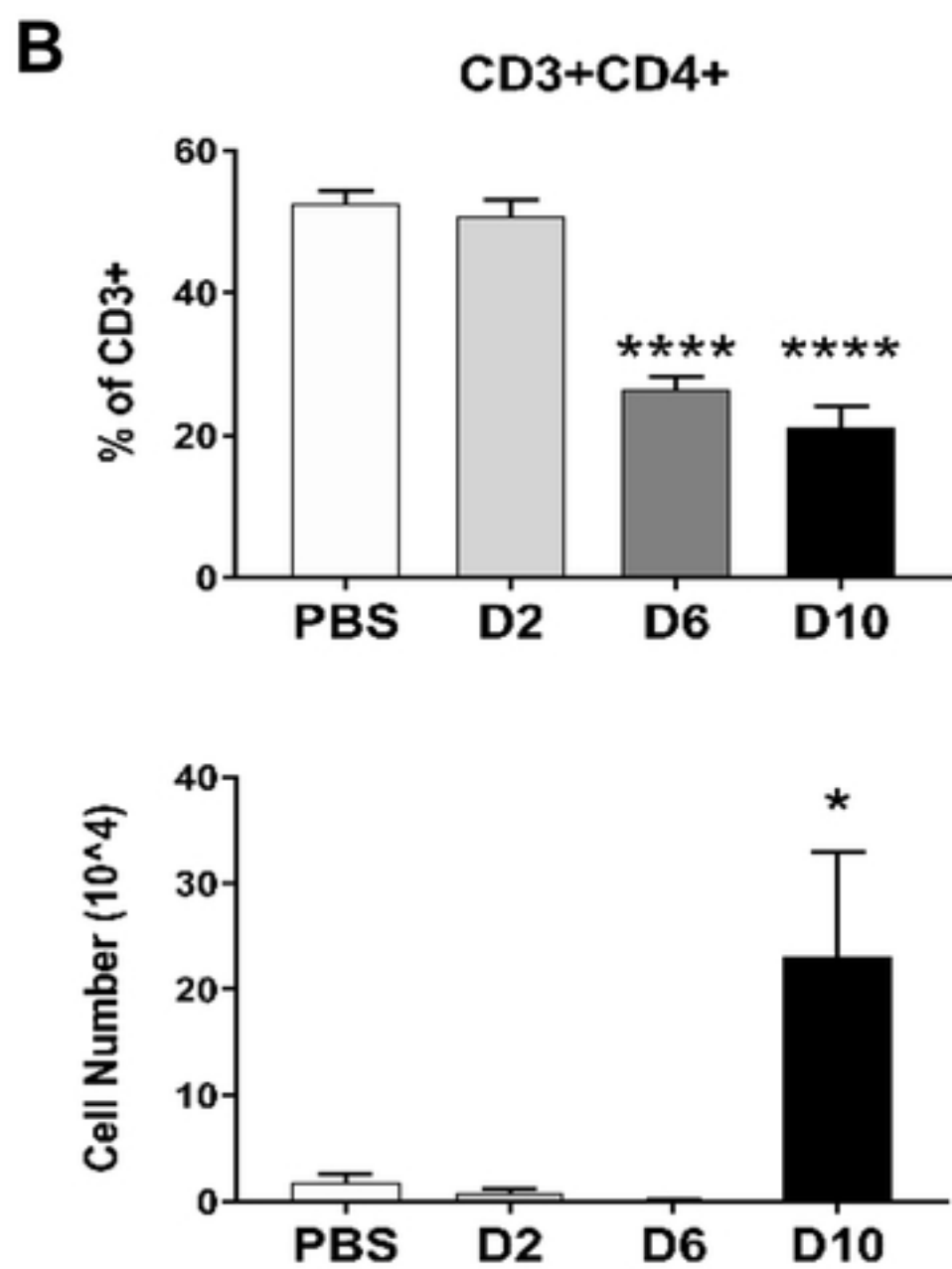
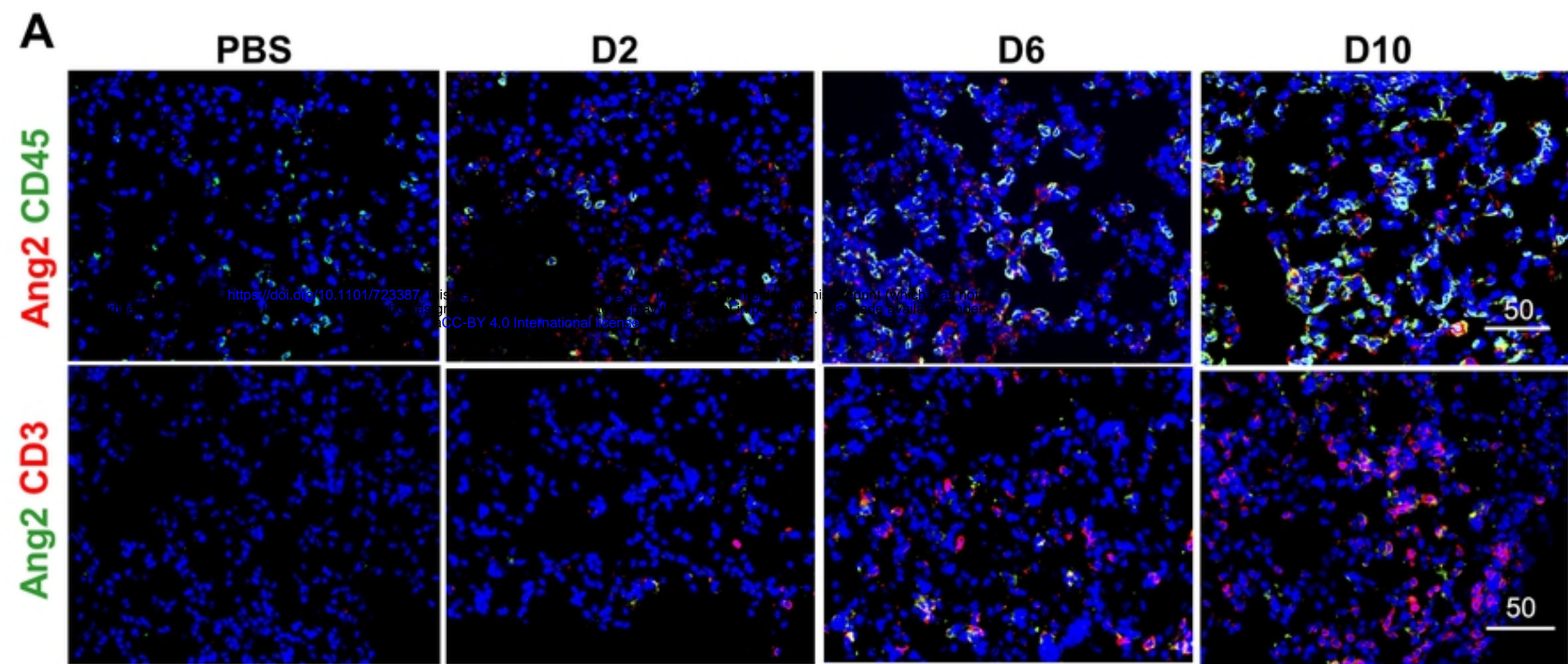
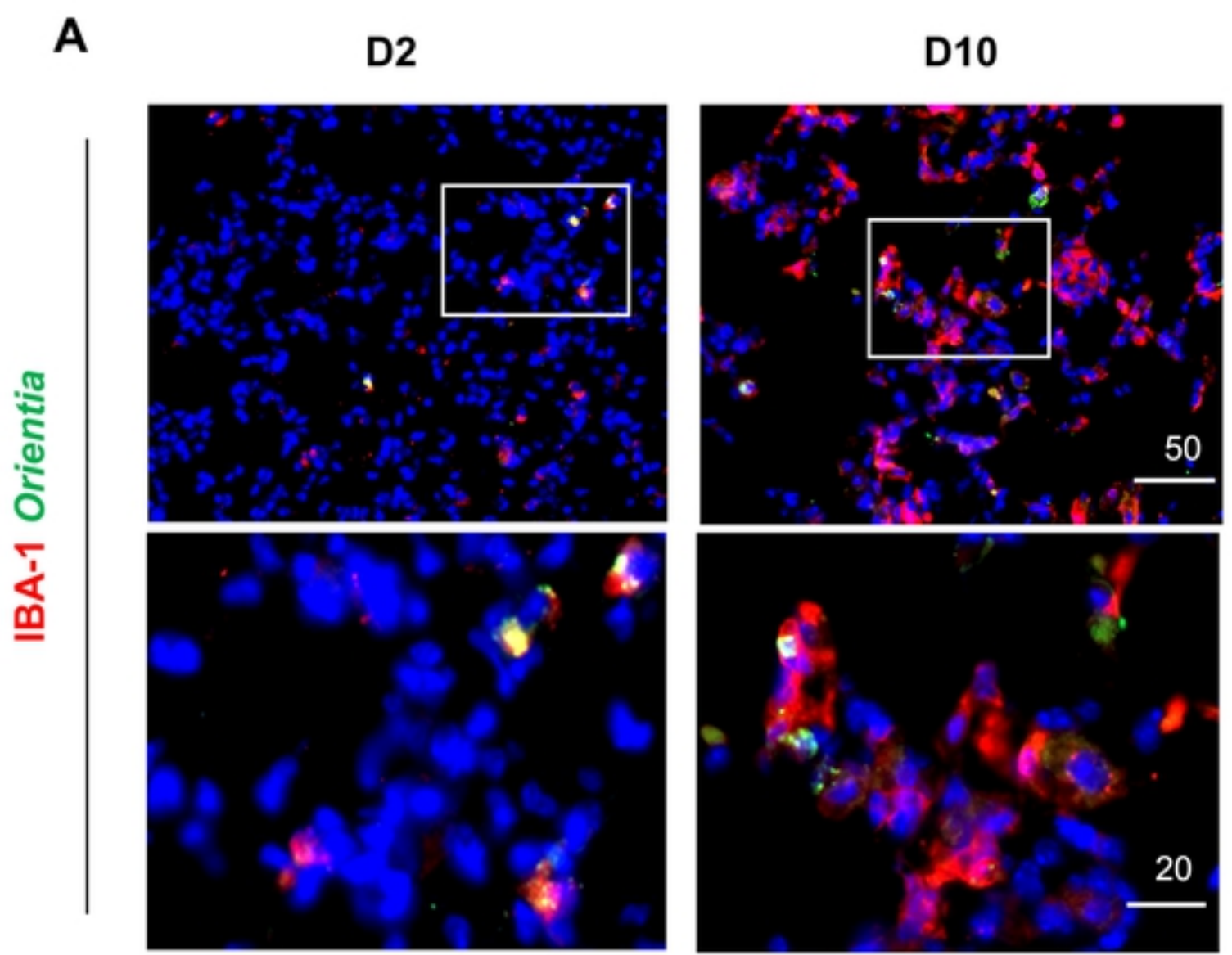


Figure S2



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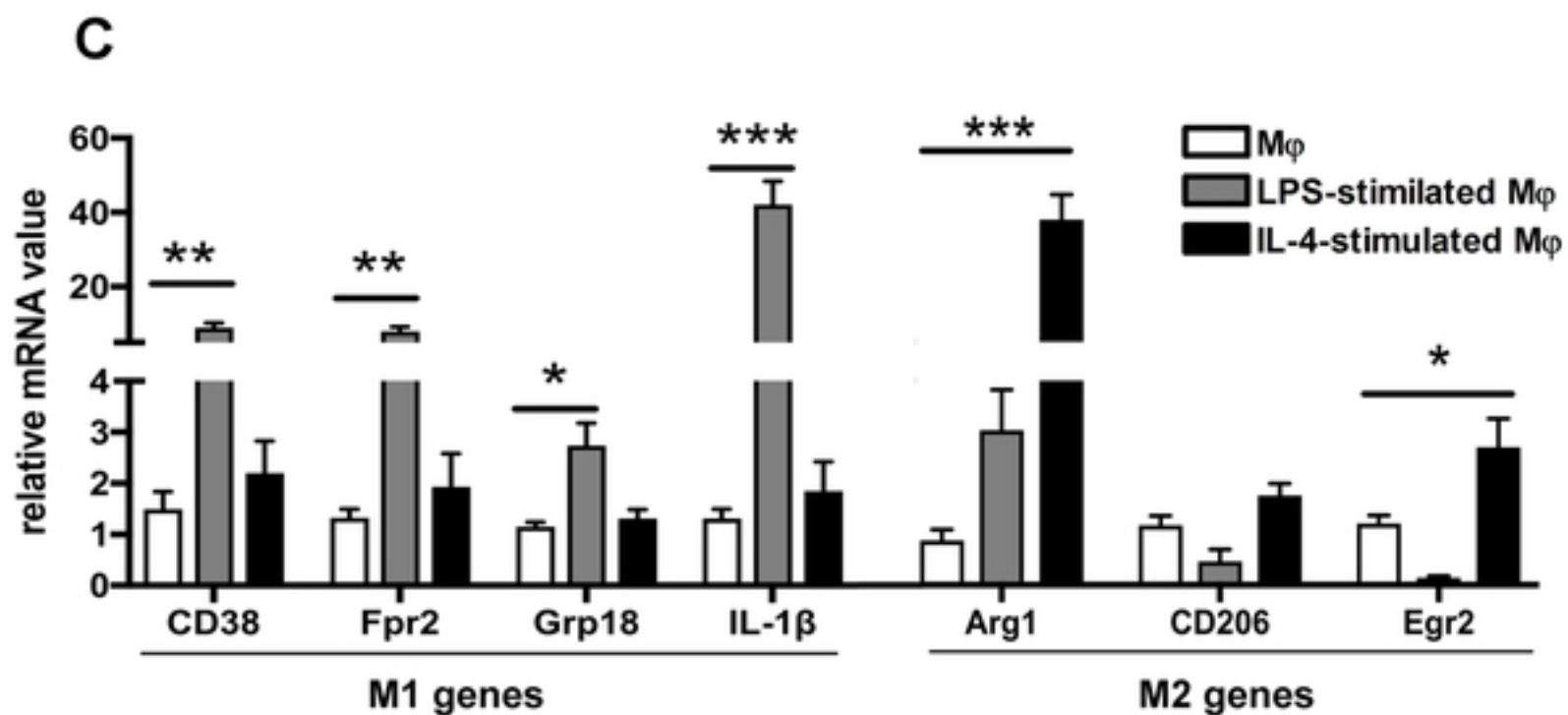
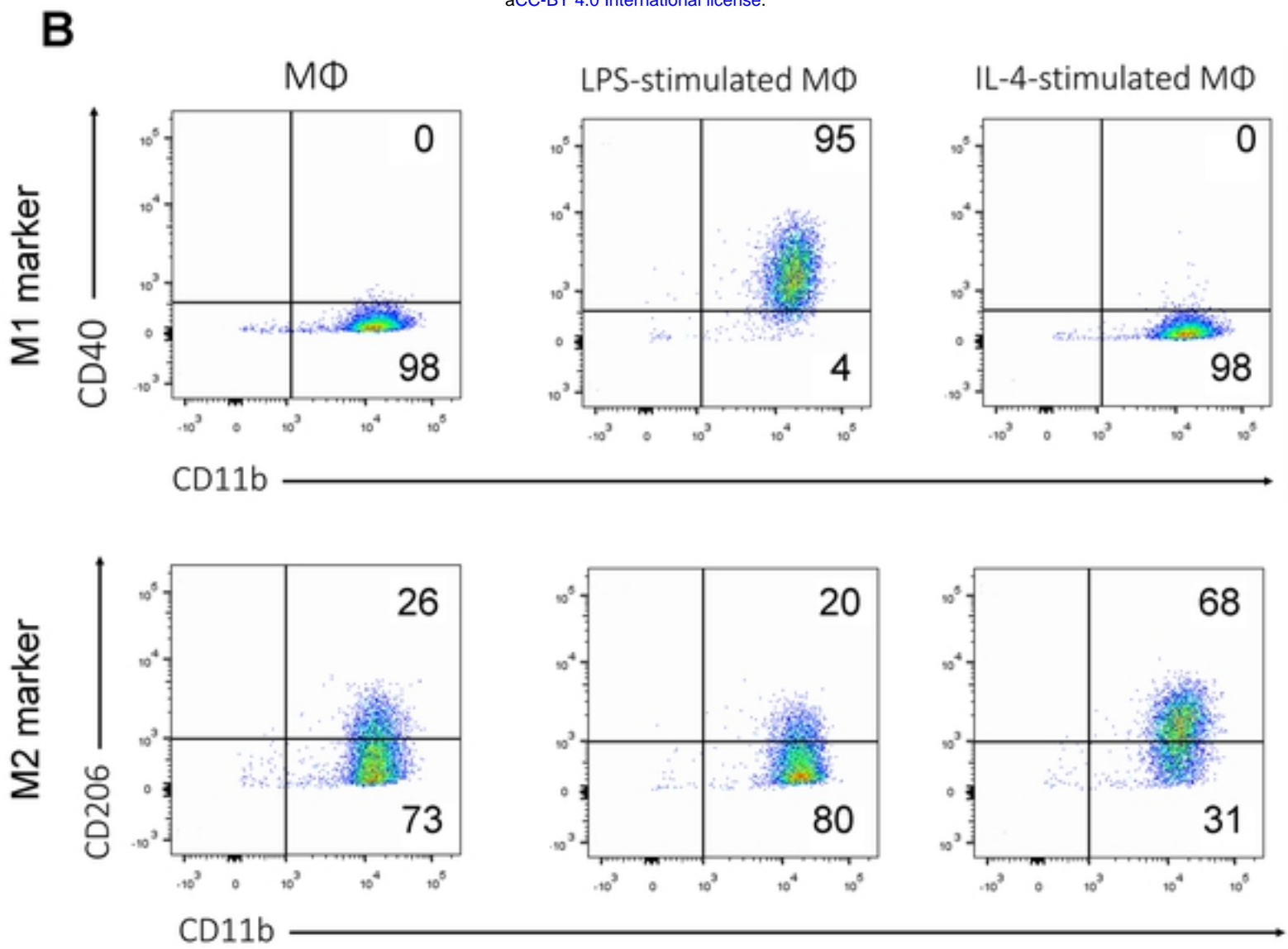


Figure S3