1   **Inducible epithelial resistance improves survival of Sendai virus pneumonia in**

2   **mice by both inactivating virus and preventing CD8^{+} T cell-mediated**

3   **immunopathology**

4   **Authors:** S. Wali[1,2], J. R. Flores[2], A.M. Jaramillo[2], D. L. Goldblatt[2], J. Pantaleón

5   García[2], M. J. Tuvim[2], B. F. Dickey[2], S. E. Evans[1,2].

6   **Author affiliations:**

7   [1]MD Anderson UTHealth Graduate School of Biomedical Sciences, Houston Texas

8   77030.

9   [2]University of Texas MD Anderson Cancer Center, Department of Pulmonary Medicine,

10   Houston Texas 77030.

11   **To whom correspondence should be addressed:** Scott E. Evans, M.D.

12   6565 MD Anderson Blvd

13   Houston TX 77030

14   Email: seevans@mdanderson.org

15   Telephone: 713-563-7433

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17   **Abstract**

18   Viral pneumonias remain a global health threat necessitating novel strategies to prevent

19   and treat these lower respiratory tract infections. We have reported that mice treated

20   with a combination of inhaled Toll-like receptor (TLR) 2/6 and TLR 9 agonists (Pam2-

21   ODN) are broadly protected against respiratory pathogens. Although a single inhalation

22   of Pam-ODN prevents acute morbidity and chronic complications associated with viral

23   pneumonias, the mechanisms underlying this protection remain incompletely elucidated.

24   Here, we show in a lethal paramyxovirus model that Pam2-ODN-enhanced survival is

25   associated with robust virus inactivation that occurs prior to internalization by lung

26   epithelial cells. However, it was also noted that viral mortality in sham-treated mice

27   temporally corresponded with CD8+ T cell-enriched lung inflammation that peaks after

28   the viral burden wanes. Pam2-ODN treatment also blocked this injurious inflammation,

29   but the attenuation of lymphocytic inflammation and the reduction in virus burden were

30   both lost when inducible reactive oxygen species generation was inhibited. Depleting

31   CD8+ T cells before or after viral challenge underscored the balanced roles of CD8+ T

32   cells in antiviral immunity and fatal immunopathology, but did not obviate the Pam2-

33   ODN antiviral protection. These findings identify multifunctional inducible antiviral

34   mechanisms and may reveal means to protect susceptible individuals against

35   respiratory infections.

36

Introduction

38   Viruses are the most frequent cause of community acquired pneumonia in children and

39   adults[1]. Respiratory viral infections result in significant morbidity and mortality in

40   vulnerable subjects, exerting a tremendous health care burden[1-4]. In addition to causing

41   acute disease, respiratory virus infections are often complicated by chronic lung

42   pathologies, such as asthma induction, progression and exacerbation[5-7]. Therefore,

2

43   development of novel therapeutic anti-viral strategies is required to effectively prevent

44   and treat respiratory infections and their associated chronic complications[8-10].

45   While lung epithelial cells are the principal targets of most respiratory viruses, there is

46   expanding evidence that lung epithelia are capable of generating anti-microbial

47   responses[7,11]. We hypothesized that lung epithelial cells can be harnessed to control

48   virus replication, thereby enhancing acute survival and reducing chronic complications

49   of virus infections[12-15]. Our group has previously described the phenomenon of inducible

50   epithelial resistance wherein the lungs' mucosal defenses can be broadly stimulated to

51   protect against a wide range of respiratory pathogens, including viruses[12-17]. This

52   protection is induced by a single inhalation of a combination treatment consisting of Toll

53   like receptor (TLR) 2/6 and 9 agonists (Pam2-ODN) before or after viral challenge.

54   While no individual leukocyte populations have been identified as critical for Pam2-

55   ODN-induced resistance, lung epithelial cells are essential to the inducible anti-viral

56   response[12]. Further, we have shown that Pam2-ODN mediated protection is dependent

57   upon epithelial generation of reactive oxygen species (ROS) but, interestingly, does not

58   require Type I interferons[16,17]. More recently, we have shown prevention of chronic

59   virus-induced asthma in mice treated with Pam2-ODN but we have not clarified the anti-

60   viral mechanisms[18].

61   In this study, we investigated the mechanisms of Pam2-ODN enhanced mouse survival

62   of paramyxovirus, Sendai virus (SeV) infection. We found that Pam2-ODN treatment not

63   only reduced lung SeV burden but also decreased epithelial cell injury and host

64   immunopathologic leukocyte responses to SeV infections. While CD8$^+$ T cells are

65   known to contribute to virus clearance, it is shown here that CD8$^+$ T cells also cause

66   substantial mortality that can be prevented by Pam2-ODN treatment. Notably, Pam2-

67   ODN-induced enhanced survival of SeV infection is effective even in CD8$^+$ T cell

68   deficient conditions. Further, we demonstrate anti-viral mechanisms of inducible

69   epithelial resistance, where virus particles are inactivated in a ROS-dependent manner

70   prior to internalization by their epithelial targets.

71   **Results**

72   **Enhanced mouse survival of SeV infection by Pam2-ODN treatment**

73   Aerosolized Pam2-ODN treatment one day prior to SeV challenge increased mouse

74   survival of SeV challenge and reduced mouse weight loss (Fig. 1a, b), similar to the

75   protection observed against lethal influenza pneumonia[12,15,16]. The survival benefit was

76   associated with reduced lung SeV burden, as measured by SeV M gene expression

77   (Fig. 1c). Investigating the natural progression of infection revealed that SeV lung

78   burden was maximal on day 5 and gradually decreased until falling below the limit of

79   quantification (LOQ) by day 11 (Fig. 1d). Pam2-ODN pretreatment reduced SeV burden

80   on all assessed days (Fig. 1d). Although the lethality of SeV infection was highly

81   dependent on the inoculum size, we found that peak mortality paradoxically occurred

82   around days 10 to 12 irrespective of inoculum size, despite the fact that SeV is

83   essentially undetectable that long after challenge (Fig. 1a, d, e). Assessing the effect of

84   Pam2-ODN on SeV burden in immortalized mouse epithelial cells (MLE-15) and primary

85   mouse tracheal epithelial cells (mTEC), we found that Pam2-ODN treatment reduced

86   SeV burden at every time point measured, reflecting the inducible antiviral capacity of

87   isolated epithelial cells (Fig. 1f).

88

Figure 1. **Pam2-ODN enhances mouse survival of SeV infection and reduces lung virus burden.** Survival **(a)** and weight loss **(b)** of mice treated with PBS or Pam2-ODN one day prior to SeV virus challenge. **(c)** Mouse lung SeV burden 5 days after infection assessed by qPCR for Sendai Matrix (M) gene (Relative quantification, RQ to 18S) relative to 18S. **(d)** Time course of lung SeV burden in mice treated with PBS or Pam2-ODN; LOQ, limit of quantification. **(e)** SeV inoculum dependent mouse survival. **(f)** SeV burden assessed by qPCR in MLE-15 cells and primary mouse tracheal epithelial cells (mTEC) treated with PBS or Pam2-ODN 4h prior to SeV challenge. n=10 mice per group in survival plots, n=4 mice/group in virus burden experiments. *$p<0.05$, **$p<0.005$.

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Pam2-ODN treatment attenuates SeV-induced epithelial injury

This temporal dissociation between peak virus burden and peak mortality led to the hypothesis that SeV-induced mortality may not be exclusively driven by excessive virus burden but may also result from untoward SeV-induced host immune response. Therefore, the acute changes in mouse lungs following SeV infection were characterized. We found increases in lung epithelial cleaved caspase 3 (cCasp3), a marker for programmed cell death, on days 7 to 11 after SeV infection (Fig. 2a, upper panel). Virus infection-related epithelial cell injury and death is typically associated with proliferative repair mechanisms[19,20]. Staining the infected mouse lung tissue for Ki67 and EdU revealed maximum signals for both markers in the second week after infection (Fig. 2b-e, upper panel). These events of lung epithelial cell death and proliferation

110   coincided with the peak of mortality (day 12, Fig. 1e). Further, hematoxylin and eosin

111   staining of lung tissues infected with SeV showed profound increases in inflammatory

112   cells from days 7 to 10 with evidence of damaged airway and parenchymal tissue (Fig.

113   2f). However, Pam2-ODN pretreatment of mice reduced epithelial cell injury and

114   proliferation (Fig. 2a-e, lower panel). This temporal association of epithelial injury and

115   death after viral clearance supported our hypothesis that mouse mortality caused by

116   SeV infection is due in part to the host immune response to SeV infections.

117

118   **Figure 2**. **Pam2-ODN pretreatment reduces epithelial cell death and proliferation**

119   **during acute SeV infection.** Cleaved caspase 3 **(a)** or Ki67 **(b)** positive cells in mouse

120   lung epithelium after SeV infection with or without Pam2-ODN treatment (lower panel).

121   EdU positive cells in axial **(c)**, small airways **(d)** and parenchyma **(e)** after SeV infection

122   with or without Pam2-ODN (lower panel). **(f)** Mouse lung histology following SeV

123   challenge with or without Pam2-ODN. n=5 mice per condition. Scale bar = 100 μm.

124   *p<0.05.

125

126   **Pam2-ODN attenuates SeV-induced lymphocytic lung inflammation.**

127   To explore this hypothesis, the host leukocyte response to SeV infection was

128   characterized. Differential Giemsa staining of bronchoalveolar lavage (BAL) cells

129   revealed increased neutrophils on days 2 to 5 and increased macrophages on days 5 to

130   8 (Fig. 3a, left and middle panel, solid grey line) after SeV challenge. Congruent with

131   our prior studies, inhaled treatment with Pam2-ODN in the absence of infection led to a

6

132 rapid rise in neutrophils that was resolved within 5 days (Fig 3a, dashed line)[21]. The

133 neutrophil response to SeV challenge was modestly increased among mice pretreated

134 with Pam2-ODN (Fig. 3a, left panel, solid dark line). Pam2-ODN-treated, SeV-

135 challenged mice showed almost no difference in macrophages compared to PBS-

136 treated, SeV-challenged mice (Fig. 3a, middle panel, solid dark line). A rise in

137 lymphocytes was observed on days 8 to 11 in PBS-treated, SeV-challenged mice (Fig.

138 3a, right panel, solid grey line), temporally corresponding with peak mortality. However,

139 Pam2-ODN treated, SeV-challenged mice displayed significantly reduced lymphocyte

140 numbers at every time point assessed (Fig 3a, right panel, solid dark line). The gating

141 strategy for lymphocyte subsets by flow cytometry is shown in Supplementary Fig. 1. A

142 modest reduction in $CD4^+$ T cells was observed in Pam2-ODN-treated, SeV-challenged

143 mice compared to PBS-treated, SeV-challenged mice (Supplementary Fig. 2). We also

144 found the percentage of $CD19^+$ $B220^+$ B cells reduced after SeV infection in comparison

145 to Pam2-ODN treated and uninfected mice (Supplementary Fig. 2), as has been seen

146 with other viral models[22,23]. However, the biggest difference between groups was in

147 $CD8^+$ T cells, with Pam2-ODN-treated, SeV-challenged mice displaying a significantly

148 lower number and percentage of $CD8^+$ T cells than PBS treated, SeV-challenged mice

149 (Fig. 3b, c). Since the greatest difference after Pam2-ODN treatment was in $CD8^+$ T cell

150 levels and there was a tight correlation between peak mortality and the increase in lung

151 $CD8^+$ T cells on days 8 to 11, we investigated the role of $CD8^+$ T cells in SeV-induced

152 mortality.

153

154 **Figure 3**. Pam2-ODN pretreatment reduces SeV induced CD8+ T cells. **(a)**

155 Differential Giemsa staining of BAL cells from mice challenged with SeV with or without

156 Pam2-ODN pretreatment. **(b)** Flow cytometry for CD8+ T cells following SeV infection

157 with or without Pam2-ODN. **(c)** Lung CD8+ T cells 11 days after SeV challenge in mice

158 pretreated with PBS or Pam2-ODN. *$p < 0.05$

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160 **CD8+ T cells contribute to anti-viral immunity but also cause lethal**

161 **immunopathology**

162 To assess the role of CD8+ T cells in SeV defense and host mortality, mouse CD8+ T

163 cells were depleted prior to SeV challenge (Fig. 4a, Supplementary Fig. 3). This

164 depletion resulted in significantly reduced survival of SeV infection (Fig. 4b). The nearly

165 90% mortality in SeV challenged CD8+ T cell depleted mice (Fig. 4b) was associated

166 with impaired viral clearance (Fig. 4c, Supplementary Fig. 4). This was not surprising,

167 given the known role of CD8+ T cells in virus clearance[24-28]. However, Pam2-ODN

168 treatment still significantly enhanced survival of SeV challenge, even in the absence of

169 CD8+ T cells (Fig. 4b). This protection was again associated with reduced SeV burden

170 compared to CD8+ T cell-depleted, SeV-challenged mice without Pam2-ODN treatment

171 (Fig. 4c, Supplementary Fig. 4). These results were congruent with our previous studies

172 showing Pam2-ODN inducible resistance against bacterial pneumonia despite the lack

173 of mature lymphocytes (*Rag1*^{-/-})[12].

174 As it appeared likely that CD8+ T cells contributed both beneficial (antiviral) and

175 deleterious (immunopathologic) effects, we depleted the CD8+ T cells on day 8 -- after

176   virus begin to clear but before peak mortality (Fig. 4d). Interestingly, mice depleted of

177   CD8+ T cells on day 8 displayed enhanced survival of SeV challenge compared to mice

178   with intact CD8+ T cells (Fig. 4e). Depletion of CD8+ T cells was confirmed by flow

179   cytometry in disaggregated lung cells 10 days after SeV challenge (Fig. 4f,

180   Supplementary Fig. 3). We also assessed lung injury by H&E staining of lung tissue 10

181   days after SeV challenge and found increased inflammation and epithelial cell damage

182   in undepleted mice compared to CD8+ T cell-depleted mice (Fig. 4g). This supported

183   our hypothesis that, while CD8+ T cells confer anti-viral immunity, they also contribute to

184   fatal SeV-induced immunopathology.

185

186   **Figure 4. Pam2-ODN treatment reduces CD8+ T cell mediated SeV induced**

187   **immunopathology.** Experimental outline **(a)**, survival **(b)** and SeV burden **(c)** 5 days

188   after SeV challenge following PBS or Pam2-ODN treatment and with or without

189   preinfection CD8+ T cell depletion. Experimental outline **(d)**, survival **(e)** and CD8+ T

190   cells **(f)** 10 days after SeV challenge following pretreatment with PBS or Pam2-ODN

191   and with or without CD8+ T cells depleted on day 8 of SeV challenge. **(g)** Lung histology

192   10 days after SeV challenged with or without Pam2-ODN treatment and/or CD8+ T cells.

193   Scale bar = 100 μm. n=10 mice/group for survival in experiment **a** and **b**; n=16

194   mice/group for survival in experiment **d** and **e**. ****$p<0.0001$ compared to PBS,

195   ***$p<0.0005$ compared to PBS, **$p<0.005$ compared to PBS, #$p<0.005$ compared to α

196   CD8 Ab-PBS, †$p<0.05$ compared to PBS, *$p<0.05$ compared to PBS, *$p<0.05$

197   compared to α CD8 Ab-PBS, †$p<0.05$ compared to α CD8 Ab-PBS.

198

Pam2-ODN treatment leads to extracellular inactivation of virus particles

199

200   As the antiviral protection consistently correlated with reduced viral burden, and as the

201   reduced virus burden likely contributes to the reduced CD8$^+$ T cell levels, we sought to

202   determine how Pam2-ODN-induced responses cause antiviral effects. We investigated

203   whether the principal Pam2-ODN effect occurred before (extracellular) or after

204   (intracellular) virus internalization into their epithelial targets. Using multiple methods to

205   determine the effect of Pam2-ODN on SeV attachment, we found no differences in

206   attachment (Fig. 5a-d). However, even though similar numbers of virus particles were

207   attached to epithelial cells, when these attached virus particles were liberated from the

208   epithelial cell targets, virus particles from Pam2-ODN-treated epithelial cells were less

209   able to subsequently infect other naive epithelial cells (Fig. 5e, f). As the number of

210   attached virus particles was the same, this difference in SeV burden in cells that

211   received liberated virus particles from PBS vs Pam2-ODN treated cells indicated that

212   SeV is inactivated prior to epithelial internalization.

213

214   **Figure 5. Pam2-ODN inhibits SeV without altering attachment**. **(a)** Flow cytometry to

215   measure virus attachment to epithelial cells. **(b)** Percentage of SeV positive epithelial

216   cells from **(a)**. **(c)** Representative examples of immunofluorescence for virus

217   attachment. **(d)** Mean fluorescence intensity of SeV-exposed epithelial cells. **(e)**

218   Experimental outline. **(f)** SeV M gene expression in MLE-15 cells (left) or primary

219   tracheal epithelial cells (right) receiving liberated virus from cultures pretreated with PBS

220   or Pam2-ODN prior to SeV infection. *$p<0.05$

221

Pam2-ODN-induced epithelial ROS protect against SeV infection and CD8^{+} T cell

immunopathology

The anti-influenza response initiated by Pam2-ODN requires epithelial generation of

ROS from both NADPH-dependent dual oxidase and mitochondrial sources[16,17].

Extending these findings to the SeV model, an NADPH oxidase inhibitor (GKT 137831)

fully abrogated the Pam2-ODN-induced anti-SeV response (Fig. 6a). Similarly,

treatment with a combination of FCCP (uncoupler of oxidative phosphorylation) and

TTFA (complex II inhibitor) obviated the Pam2-ODN-induced anti-SeV response (Fig.

6b)[16,17]. Further, it was found that Pam2-ODN induced epithelial generation of ROS

were required for inactivation of SeV prior to epithelial entry (Fig. 6c). Congruent with

these *in vitro* and *ex vivo* studies, mice treated with FCCP-TTFA before Pam2-ODN

treatment and SeV challenge (Fig. 6d) demonstrated reduced survival (Fig. 6e),

increased SeV burden (Fig. 6f), and increased CD8^{+} T cells on day 10 (Fig 6g).

235

Figure 6. **Pam2-ODN induced reactive oxygen species protects against acute SeV**

virus infections and immunopathology. SeV burden in MLE-15 cells with or without

treatment with Pam2-ODN and/or NADPH inhibitors **(a)** or mitoROS inhibitors **(b)**. **(c)**

SeV M gene expression in MLE-15 cells receiving liberated virus from cells pretreated

with PBS/Pam2-ODN with or without mitoROS inhibition. **(d)** Experimental outline. **(e)**

Survival of SeV challenge in mice treated with PBS or Pam2-ODN and/or mtROS

inhibitors. **(f)** Lung SeV burden measured on day 5 and **(g)** lung CD8^{+} T cells assessed

243   on day 10. n=13 mice/group in experiment **d** and **e**. ***p<0.0001, **p<0.005, **p<0.01

244   compared to PBS, †*p*<0.05 compared to Pam2-ODN, *p<0.05, *p<0.01.

245

Discussion

247   In this study, we demonstrate that therapeutic stimulation of lung epithelial cells

248   enhances mouse survival of acute SeV infections by both reducing the virus burden and

249   attenuating host immunopathology. While our group has demonstrated inducible

250   resistance against multiple respiratory pathogens including viruses[12-17,21], these studies

251   demonstrate for the first time when in the virus lifecycle the anti-viral effects begin,

252   substantiate the role of ROS in SeV protection, and reveal protective

253   immunomodulatory effects.

254   While Pam2-ODN treatment provided a significant host survival benefit in SeV infection,

255   we observed this survival difference occurring even after the PBS-treated mice had

256   cleared the virus. This observation prompted the hypothesis that host mortality is not the

257   exclusive result of direct viral injury to the lungs, but due at least in part to the host

258   response to the virus infections. Eliminating the anti-viral component of CD8^{+} T cells in

259   this model, we observed enhanced survival of SeV infections in mice depleted of CD8^{+}

260   T cells on day 8 (Fig. 4), revealing the importance of balancing the dual functions of

261   CD8^{+} T cells in anti-viral immunity and in causing fatal immunopathology. Our findings

262   suggest that the surge in CD8^{+} T cells within the lungs after most virus has been

263   cleared causes physiologic impairment via lung injury and cell death (Fig 4d-g).

264   However, Pam2-ODN treatment enhanced survival of SeV infections, reflecting the

265   intrinsic anti-viral capacity of the lung epithelial cells. This finding is potentially valuable

266   in the context of treating pneumonia in immunocompromised patients.

267   Previous reports support this concept of counter-balanced immune protection and

268   immunopathology by CD8^{+} T cells during virus infections[24,28-32]. Some reports have

269   shown that antigen-experienced memory CD8^{+} T cells enhance respiratory syncytial

270   virus (RSV) clearance, but also mediate severe immunopathology[30,33]. However, our

271   study is the first to demonstrate the survival advantage of paramyxovirus infections by

272   either temporal depletion of enhanced CD8^{+} T cells or by inducible epithelial resistance.

273   Our findings are also congruent with reports on the role of CD8^{+} T cells in non-

274   respiratory viral infection models, such as in West Nile virus infection, where CD8^{+} T cell

275   deficient mice display decreased mortality[31]. Findings from this study and others reveal

276   that the harmful effects of CD8^{+} T cell mediated immunopathology may supersede the

277   benefits of T cell mediated viral clearance. Therefore, it is appealing to develop

278   inducible anti-microbial strategies that do not rely on conventional T cell-mediated

279   microbial clearance and are also effective in vulnerable immune deficient

280   populations[12,16,34,35].

281   Although the CD8^{+} T cell depletion studies enhanced our understanding of

282   immunopathology in virus infections, much of the survival benefit against SeV infection

283   was presumably mediated by anti-viral effects induced by Pam2-ODN. This led us to

284   investigate the mechanisms of these inducible anti-viral effects. Given the multiple steps

285   in the virus life cycle, it was not known at what stage Pam2-ODN exerted its anti-viral

286   effect. Exploring this, we found no difference in number of SeV particles attached to the

287   cells between PBS and Pam2-ODN treatment (Fig 5a-d). However, attached virus

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288   particles that were from Pam2-ODN treated cells retain less infective capacity when

289   added to naïve epithelial cells, revealing pre-internalization virus inactivation by Pam2-

290   ODN treatment (Fig 5e, f).

291   Knowing that Pam2-ODN inducible resistance required ROS production to protect

292   against influenza[16], we studied the role of ROS in Pam2-ODN-mediated reduction in

293   SeV burden. ROS inhibition not only led to attenuation of Pam2-ODN's anti-viral effect

294   but was also permissive for increased lung CD8+ T cell numbers *in vivo*, implicating

295   Pam2-ODN-induced ROS in preventing both identified mechanisms of mouse mortality

296   in SeV pneumonia (Fig 6a, b, e, f). Further, ROS inhibition also led to loss of Pam2-

297   ODN-inducible *in vitro* inactivation of SeV prior to epithelial internalization (Fig. 6c),

298   demonstrating for the first time that epithelial ROS directly contribute to virus

299   inactivation.

300   Production of ROS as a microbicidal mechanism has been widely reported in

301   phagocytic cells[36-38]. However, this mechanism has not been extensively studied in non-

302   phagocytic cells[39], where it apparently acts predominantly extracellularly rather than

303   intracellularly as in phagocytes. (Fig. 5f, g). Induction of a sustained microbicidal state

304   through lung epithelial reprogramming can potentially explain the broad protection seen

305   against multiple pathogens[12-14,21,34,40]. These findings of viral inactivation by epithelial

306   ROS production reveal an essential component of inducible epithelial resistance.

307   Taken together, these findings provide mechanistic insights into the role of the lung

308   epithelium in induction of anti-viral responses and prevention of host immunopathology

309   that may inform future therapeutics to protect vulnerable populations.

Methods

Mice: All *in vivo* experiments were performed using 6 to 10-week-old C57BL/6J mice of a single sex purchased from (Jackson laboratory) or bred in-house according to the Institutional Animal Care and Use Committee of MD Anderson Cancer Center, protocol 00000907-RN01.

Cells: Mouse lung epithelial (MLE-15) cells were kindly provided by Jeffrey Whitsett, Cincinnati Children's Hospital Medical Center, and cultured in DMEM with 2 % Fetal Bovine Serum (FBS), 1 % insulin and transferrin. MLE-15 cells were authenticated by the MD Anderson Characterized Cell Line Core Facility. To harvest tracheal epithelial cells, mice were anesthetized to expose and excise tracheas. These tracheas were then digested in pronase (1.5 mg/ml, Sigma Aldrich) overnight at 4⁰ C. Tracheal epithelial cells were then isolated and cultured on collagen coated transwells in Ham's F12 media supplemented with differentiation growth factors and hormones as described previously[16,35].

TLR treatments and viral challenge: For *in vitro* treatments, cells were treated with Pam2 (2.2 μM) and ODN (0.55 μM), 4 h before SeV inoculation as previously described[16,17]. For *in vivo* treatments, 10 ml solution of Pam2 (4 μM) and ODN (1 μM) in endotoxin free water was delivered by Aerotech II nebulizer (Biodex Medical Systems) driven by 10 l/min along CO_2 (5 %) in air for 30 minutes as previously described[16,17]. SeV was purchased from ATCC (Manassas, VA) and grown in Rhesus monkey cells obtained from Cell Pro labs (Golden Valley, MN). For *in vitro* inoculations, multiplicity of infection (MOI) of one was used. Unless otherwise stated, mice were challenged with 1 x 10^8 plaque forming units (pfu) in PBS inserted into the oropharynx of mice, under

15

333   isoflurane anesthesia as described[18]. Mice were weighed before and daily after

334   challenge as a measure of morbidity and criteria for euthanasia.

335   **Bronchoalveolar lavage and differential Giemsa staining**: Mouse tracheas were

336   instilled with 1.5 ml of PBS through a 20-gauge cannula after deep sedation and

337   approximately 1 ml of BAL fluid was collected. The BAL fluid was then spun down at 4^0

338   C at 300 g to collect the cells in the pellet. The cell pellet was resuspended in 1 ml of

339   ice-cold PBS and 200 μl of this cell suspension was then subjected to cytocentrifugation

340   at 300 g for 5 min. Cells were stained with Giemsa stain for differential counts

341   determination and total cells were counted by hemocytometer.

342   **Flow cytometry**: For *in vivo* experiments, mouse lungs were perfused with 5 to 10 ml

343   PBS, dissected, cut into 1 mm^3 pieces, and digested with collagenase/DNAse I (5

344   mg/ml, Worthington biochemical) for 30 min at 37° C. After digestion, single cells were

345   collected by passing through a 70 μm filter. These single cells were washed with FACS

346   staining buffer (PBS supplemented with 1 % FBS) and stained for specific cell types, as

347   indicated in the antibody table (Table 1). For *in vitro* experiments, MLE-15 cells were

348   seeded on 24 well plates for treatment with Pam2-ODN and SeV inoculation. Cells were

349   trypsinized and washed with FACS staining buffer 2X. Cells were blocked in 5 %

350   donkey serum for 30 min before proceeding to staining with Rabbit SeV antibody (MBL

351   International) overnight at 4° C, followed by staining with secondary Alexa488 anti-rabbit

352   antibody (Jackson Immunologicals) for 1 h. Cells were fixed and acquired on BD LSRII

353   (BD Biosciences) for Alexa488 positive cells.

354   **Immunofluorescence:** MLE-15 cells were grown on chamber slides (Labtek), treated

355   with Pam2-ODN for 4 h before inoculation with SeV (MOI 1). Cells were then fixed with

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356   2 % paraformaldehyde before staining with Rabbit SeV antibody (MBL International)

357   and detected using a secondary anti-rabbit antibody. For each experimental condition,

358   specimens were imaged using Olympus BX 60 microscope using identical parameters

359   for time of exposure, color intensity, contrast and magnification. Images were then

360   loaded on ImageJ software to calculate mean fluorescence intensity for each group.

361   **H&E staining:** Mouse lungs were fixed by intratracheal inflation with 10 % formalin for

362   24 h, and then transferred to 70 % ethanol embedded in paraffin. Tissue blocks were

363   then cut into 5 μm sections, mounted onto frosted glass slides, deparaffinized with

364   xylene, washed with ethanol, then rehydrated and stained with hematoxylin and eosin

365   for morphological changes.

366   **Epithelial proliferation assays:** Mice were injected intraperitoneally with 0.1 ml EdU (1

367   mg/mouse). After 24 h, lungs were inflated and fixed with 10 % formalin for 24 h at 4° C

368   and then lungs were embedded in paraffin. Paraffin sections were cut into 5 μm

369   transverse sections of the axial airway, between lateral branches 1 and 2. Lung sections

370   were then stained following the Click-iT EdU Imaging Kit protocol for EdU (Abcam,)

371   followed by staining with DAPI for 30 min at room temperature. Images were collected

372   using Olympus BX 60 microscope using identical parameters for all conditions. Some

373   lung sections were subjected to antigen retrieval and then stained for Ki67 (1:1000;

374   Invitrogen) and cCasp3 (1:500; Cell Signaling). EdU, Ki67 or cCasp3 positive cells were

375   quantified using cell counter plugin in ImageJ and normalized to DAPI positive cells in

376   every field of view (number of fields surveyed per mouse sample = 3).

377

378 **CD8+ T cell depletion**: Anti CD8-β antibody (200 µg/mouse, Bioxell) was delivered to

379 mice intraperitoneally at indicated time points. CD8+ T cell depletion was confirmed by

380 flow cytometry analysis 24 to 48 h after depletion.

381 **Viral burden quantification**: For *in vivo* experiments, mouse lungs were collected 5

382 days after SeV challenge. RNA from mouse lungs was extracted using the Qiagen

383 RNeasy kit. 500 ng of total RNA was converted to cDNA using Biorad iScript cDNA

384 conversion kit. Viral burden was determined by reverse transcription quantitative PCR

385 (RT-qPCR) of the Sendai Matrix (M) protein normalized to house-keeping gene

386 18SRNA. 18S forward primer – GTAACCCGTTGAACCCCATT; reverse primer –

387 CCATCCAATCGGTAGTAGCG. SeV M gene forward primer –

388 ACTGGGACCCTATCTAAGACAT; reverse primer – TAGTAGCGGAAATCACGAGG.

389 The Limit of quantification (LOQ) was established for the SeV qPCR assay as the

390 highest dilution of the template still maintaining the linearity of the assay. The threshold

391 cycle (CT) value of the LOQ was set as the lower limit for the assay.

392 **ROS inhibition *in vitro* and *in vivo*:** NADPH oxidase activity was inhibited by exposing

393 the cells to GKT137831 (10 µM; Selleckchem) 12 h prior to treatment with Pam2-ODN

394 or PBS. Mitochondrial ROS production was inhibited using the combination of FCCP

395 (400 nM, Cayman Chemicals) and TTFA (200 µM, Cayman Chemicals) for 1 h before

396 Pam2-ODN or PBS treatment. For *in vivo* experiments, mice were aerosolized with

397 10 ml TTFA (200 mM) and FCCP (800 µM) 2 h before Pam2-ODN aerosolization and 2

398 h before SeV challenge and then again 4 days after SeV challenge[16].

399 **Viral attachment assays:** MLE-15 cells were cultured in 24 well plates or chamber

400 slides for treatment with Pam2-ODN and SeV inoculations. Cells were placed on ice 30

18

401   min before inoculation with SeV to prevent viral entry into the cells. 4 h after inoculation

402   on ice, cells were vigorously washed 5X with media to remove unattached virus. Cells

403   were then harvested to measure SeV burden using immunofluorescence or flow

404   cytometry. For RT-qPCR assays, epithelial cells were treated with Pam2-ODN or PBS,

405   followed by SeV infection on ice (to prevent viral entry into cells). Virus particles were

406   allowed to attach to the epithelial targets for 4 h on ice. These cells were then

407   extensively washed to remove unattached virus particles, and then the cells were lysed

408   by passing through a syringe 10X. The liberated virus particles were then transferred to

409   naïve epithelial cells that had no prior exposure to Pam2-ODN. SeV M gene expression

410   was assessed by qPCR after 24 h of SeV replication in the new cells. The experimental

411   design is illustrated in Fig 5e. In some experiments, mitoROS inhibitors (FFCP-TTFA)

412   were used before Pam2-ODN treatment to determine the role of Pam2-ODN induced

413   ROS in SeV inactivation prior to internalization.

414   **Statistics:** All statistical analysis was performed using GraphPad Prism software

415   (Version 8 for Windows, GraphPad Software, La Jolla, CA). To determine pairwise

416   differences in viral burden or cell numbers, Student's t test was used. Mouse survival

417   analysis of viral challenges were analyzed using Mantel-Cox test. One-way analysis of

418   variance (ANOVA) with multiple comparisons was used to determined differences

419   between multiple experimental conditions.

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423 **Supplementary materials:**

424 **Supplementary Figure 1.** Gating strategy for flow cytometry of lung T and B cells.

425 **Supplementary Figure 2.** Pam2-ODN pretreatment reduced SeV induced

426 lymphocytes. Disaggregated mouse lung cells positive for CD4$^+$ T cells, CD19$^+$ B220$^+$ B

427 cells, CD8$^+$ T cells assessed by flow cytometry in perfused lungs from mice treated with

428 PBS or Pam2-ODN on various days of SeV challenge. *p<0.05 compared to PBS+SeV,

429 **p<0.01 compared to PBS+SeV.

430 **Supplementary Figure 3.** CD8$^+$ T cell depleting antibody reduced lung CD8$^+$ T cells.

431 **Supplementary Figure 4**. Lung SeV burden 3, 5 and 7 days after SeV challenge in

432 mice treated with PBS or Pam2-ODN with or without CD8$^+$ T cell depletion. *p<0.05

433 compared to PBS treated group.

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444   **Table 1**

Antibodies	Vendor	Catalogue numbers
CD3	Tonbo	65-0031-U100
CD4	Tonbo	60-0042-U100
CD8	Tonbo	25-0081-U100
Live dead	Tonbo	13-0870-T500
CD25	Biolegend	102038
Foxp3 Treg kit	eBiosciences	72-5775
CD8-Depleting Ab	Bioxell	BE0223-A025
CD19	Biolegend	115507
B220	BD Biosciences	562922
Anti-SeV virus Ab	MBL International	PD029
Ki67	Invitrogen	MA5-14520
cCasp3	Cell signaling	9662S

445

446   **Author contributions:**

447   S.W. designed and performed the experiments, analyzed the data, and wrote the

448   manuscript. J.R.F., A.M.J., D.L.G. and J.P.G performed experiments. M.J.T., B.F.D.

21

449   conceptualized the project and critically reviewed the data. S.E.E. conceptualized the

450   project, designed experiments, provided critical evaluation of data and edited the

451   manuscript.

452   **Acknowledgments:**

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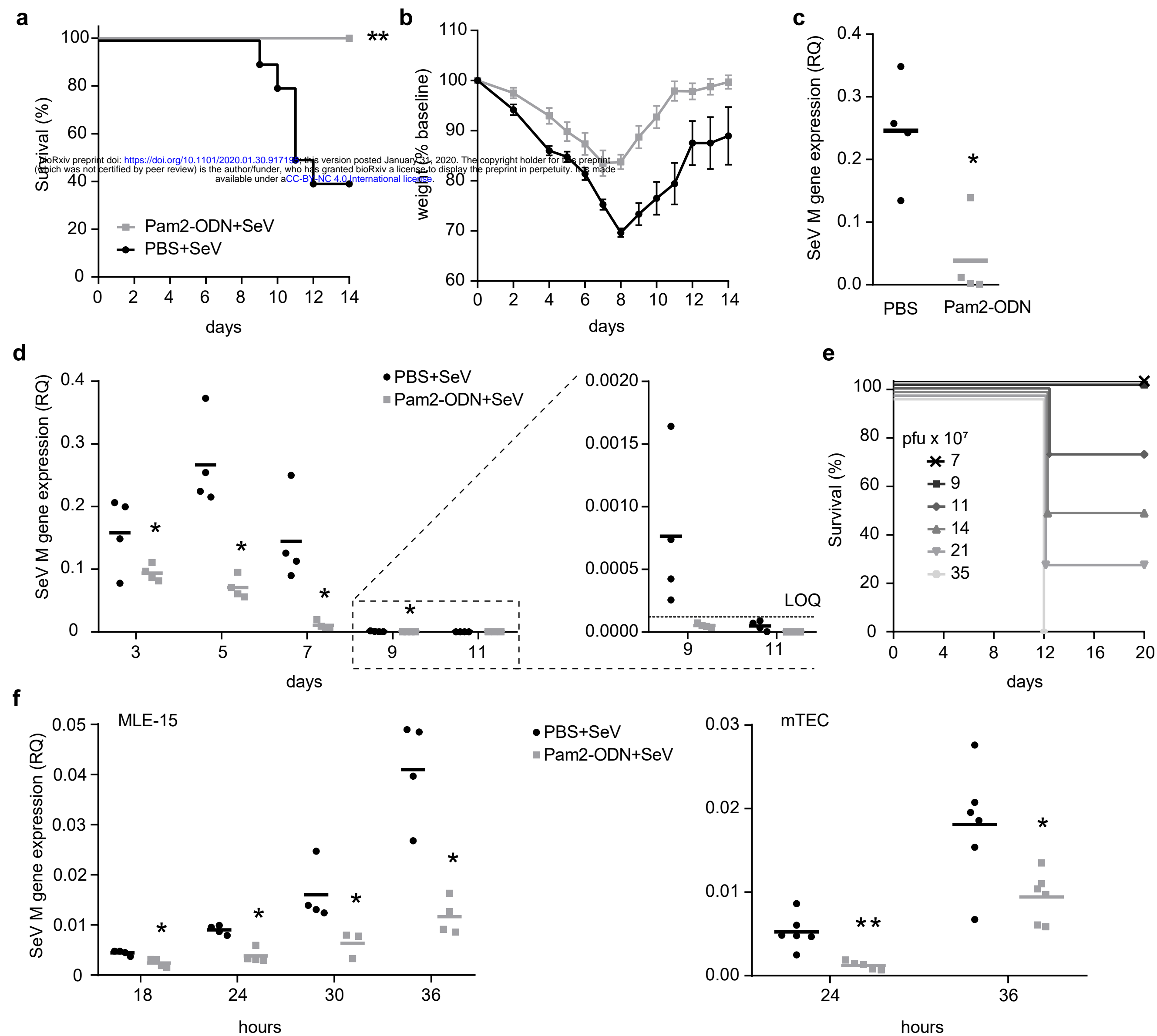
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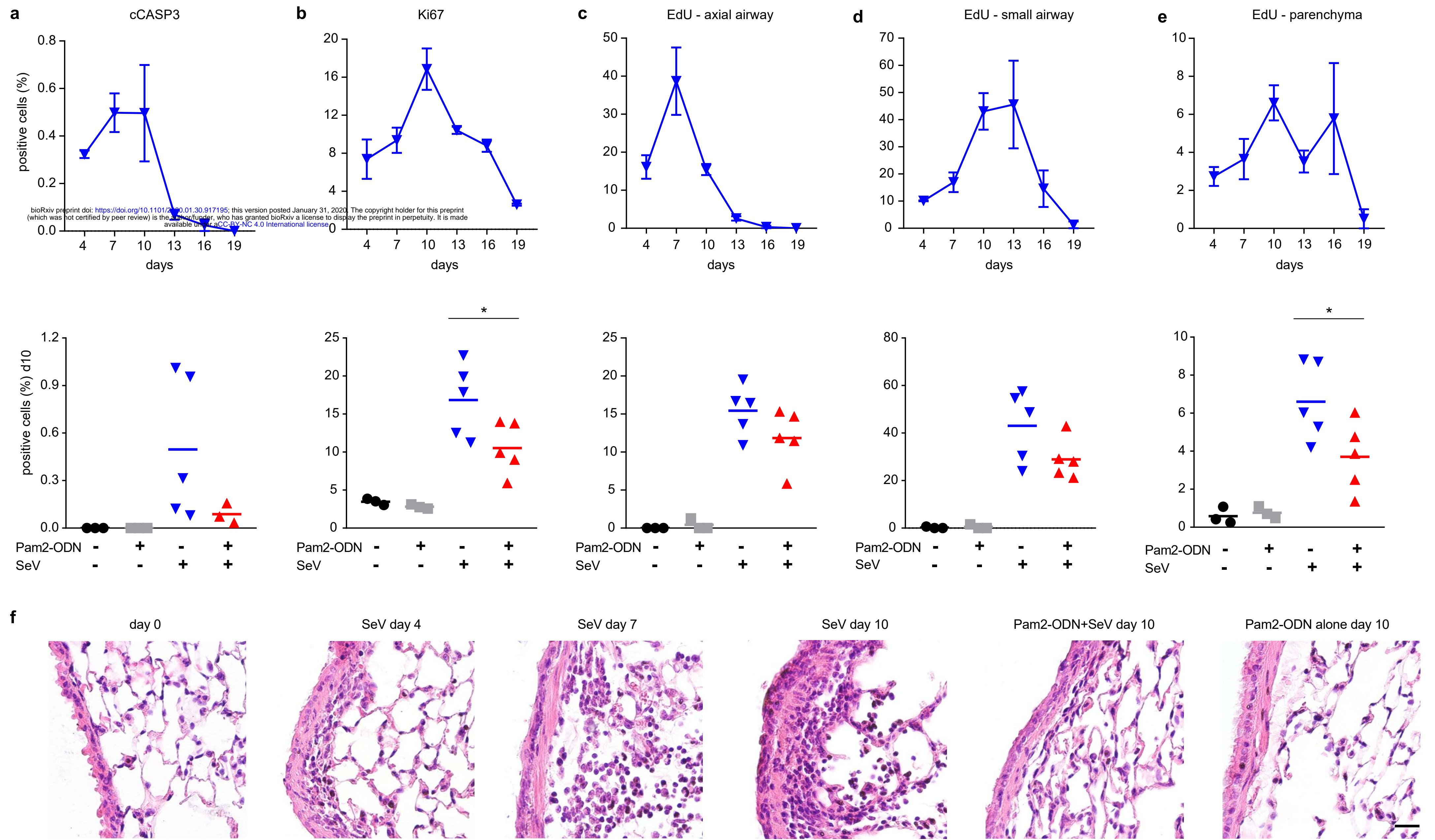
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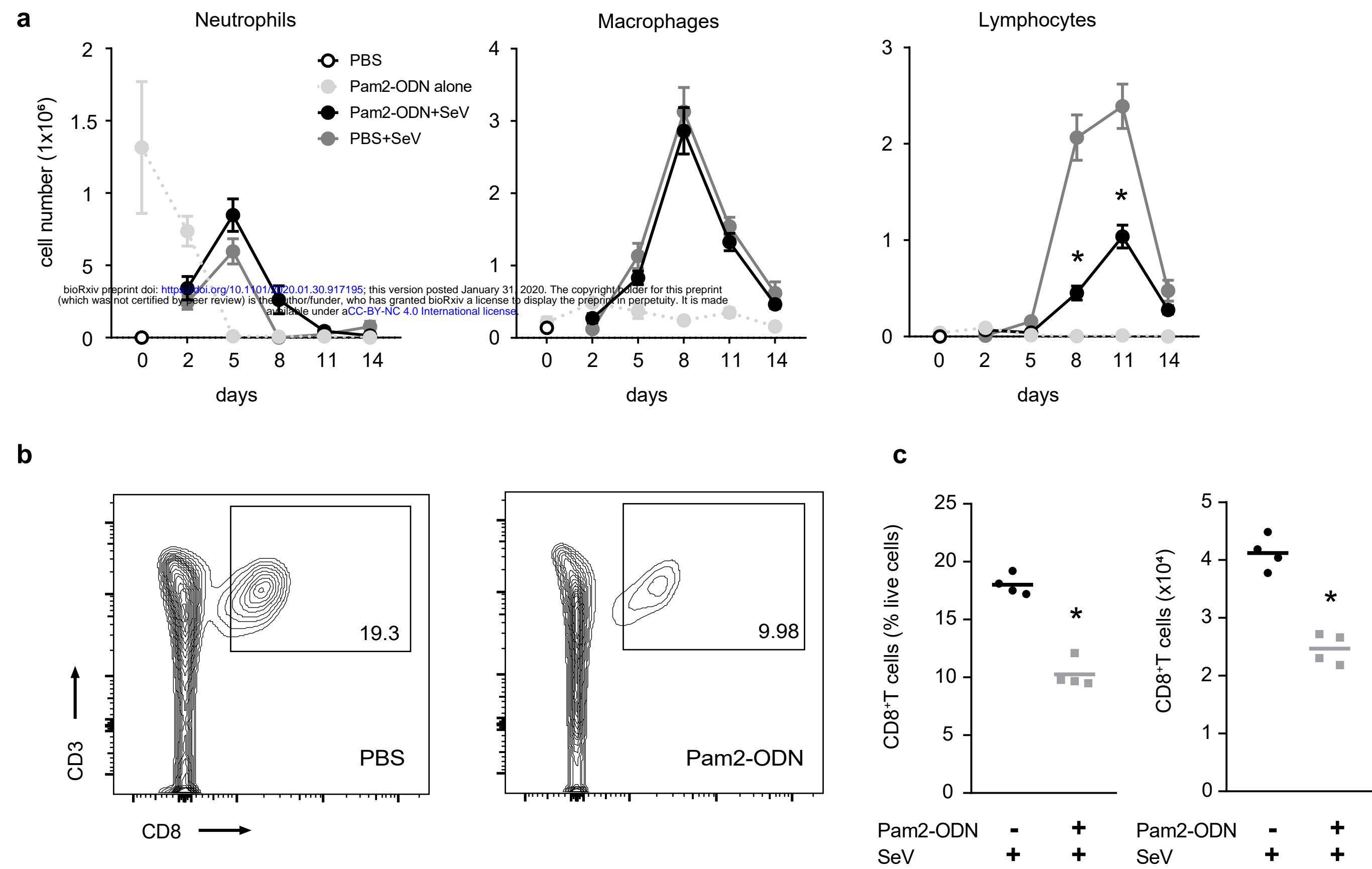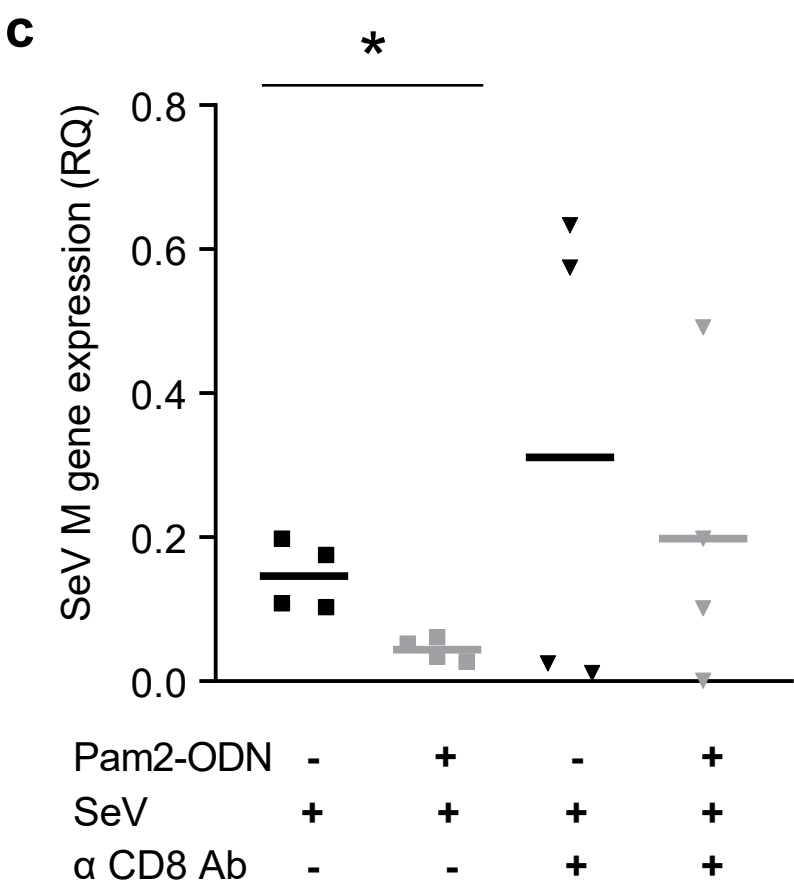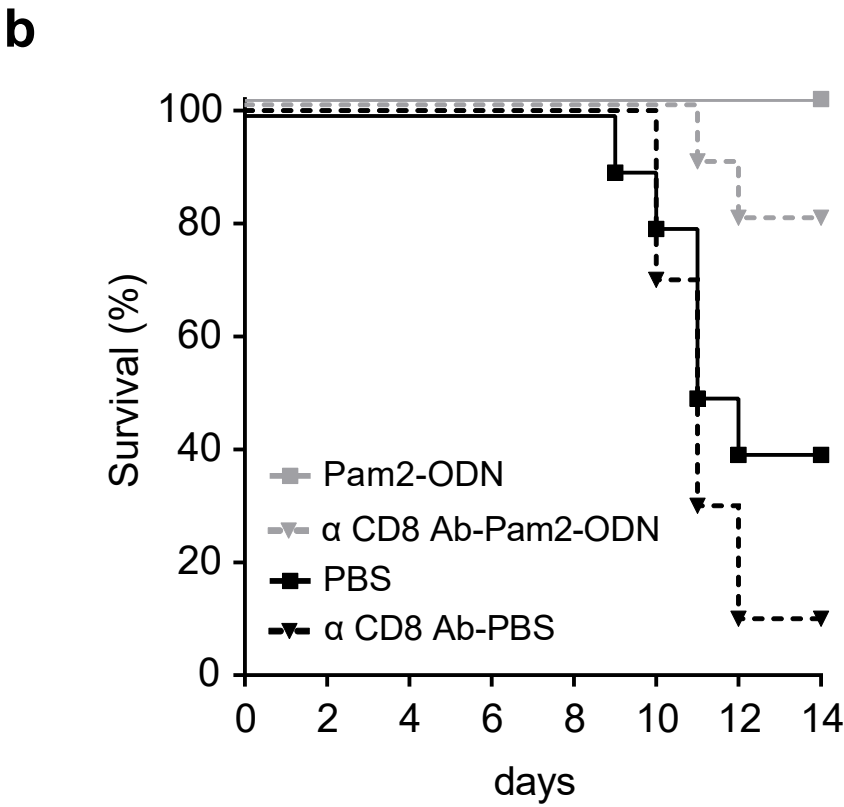
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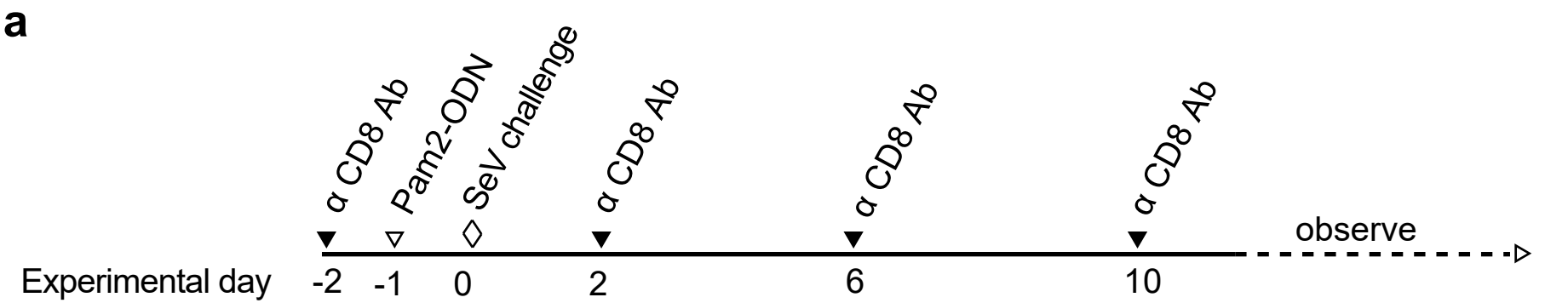**Wali, Figure 1**

Wali, Figure 2

a cCASP3

b Ki67

c EdU - axial airway

d EdU - small airway

e EdU - parenchyma

f day 0 | SeV day 4 | SeV day 7 | SeV day 10 | Pam2-ODN+SeV day 10 | Pam2-ODN alone day 10

Wali, Figure 3

a

Neutrophils

Macrophages

Lymphocytes

○ PBS
○ Pam2-ODN alone
● Pam2-ODN+SeV
● PBS+SeV

cell number (1x10⁶)

days

b

CD3

CD8

19.3

PBS

9.98

Pam2-ODN

c

CD8⁺T cells (% live cells)

CD8⁺T cells (x10⁴)

| Pam2-ODN | − | + |
| SeV | + | + |

| Pam2-ODN | − | + |
| SeV | + | + |

Wali, Figure 4

a

α CD8 Ab | Pam2-ODN | SeV challenge | α CD8 Ab | α CD8 Ab | α CD8 Ab | observe

Experimental day: -2 -1 0 2 6 10

b

Survival (%) vs days

- Pam2-ODN
- α CD8 Ab-Pam2-ODN
- PBS
- α CD8 Ab-PBS

** # †

c

SeV M gene expression (RQ)

*

Pam2-ODN	-	+	-	+
SeV	+	+	+	+
α CD8 Ab	-	-	+	+

d

Pam2-ODN | SeV challenge | α CD8 Ab | Flow cytometry | observe

Experimental day: -1 0 8 10

e

Survival (%) vs days

- Pam2-ODN
- α CD8 Ab-Pam2-ODN
- PBS
- α CD8 Ab-PBS

*** † *

f

CD8+ T cells (% live cells)

**** **** ***

Pam2-ODN	-	+	-	+
SeV	+	+	+	+
α CD8 Ab	-	-	+	+

g

PBS | Pam2-ODN | α CD8 Ab-PBS | α CD8 Ab-Pam2-ODN

Wali, Figure 5

a

PBS

Pam2-ODN

Events

3.55

3.72

ns

α SeV Ab

b

ns

positive cells (%)

Pam2-ODN   −   +
SeV   +   +

c

PBS

Pam2-ODN

DAPI Sendai

d

ns

MFI (A.U.)

Pam2-ODN   −   +
SeV   +   +

e

PBS or
Pam2-ODN

Transfer lysate to
naïve MLE-15 cells

qPCR

MLE-15 cells

3.5h

0.5h

4h

24h

Place
on ice

SeV

Wash 10x and
lyse cells

f

MLE-15

mTEC

SeV M gene expression (RQ x 10-3)

*

*

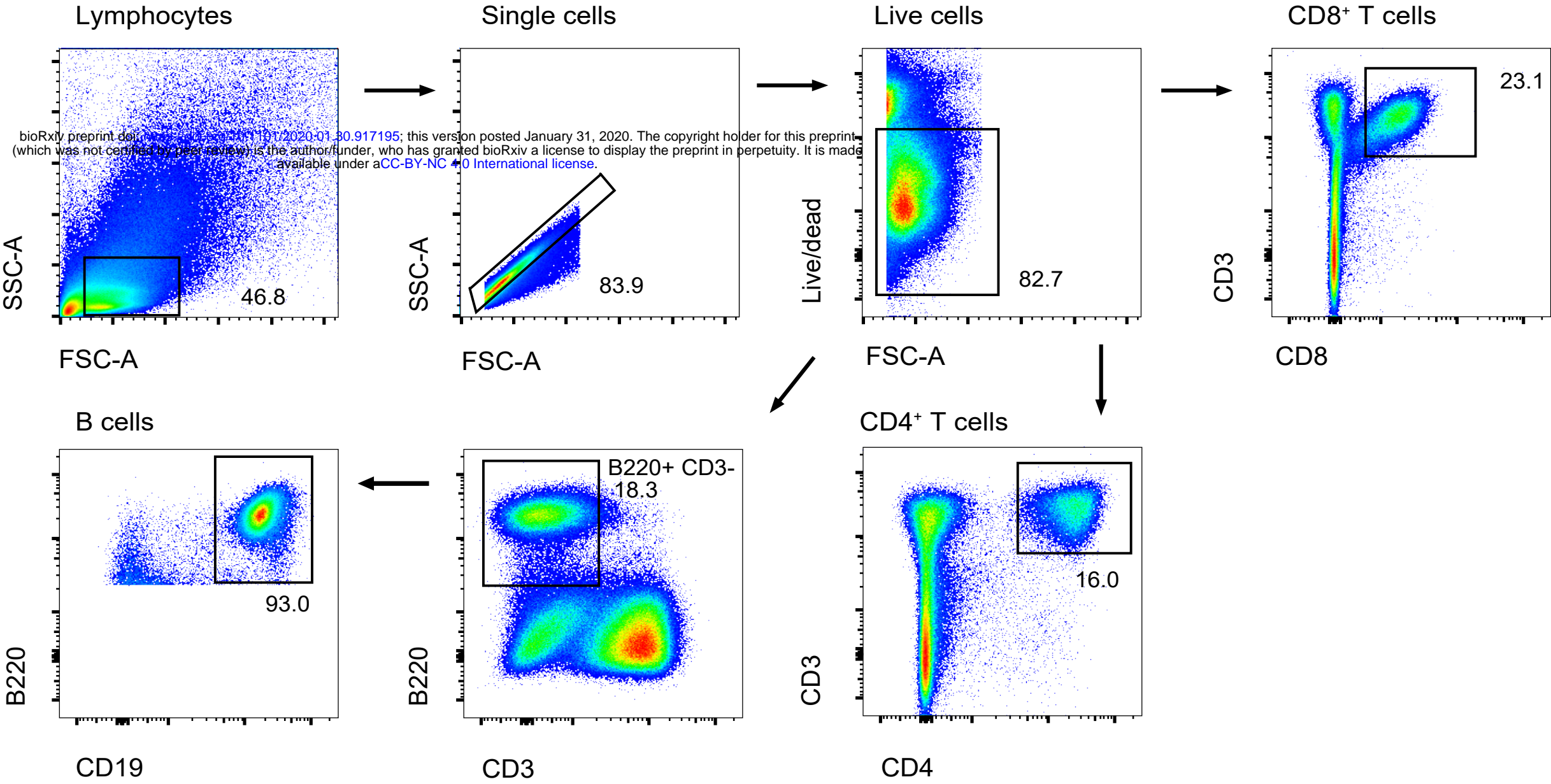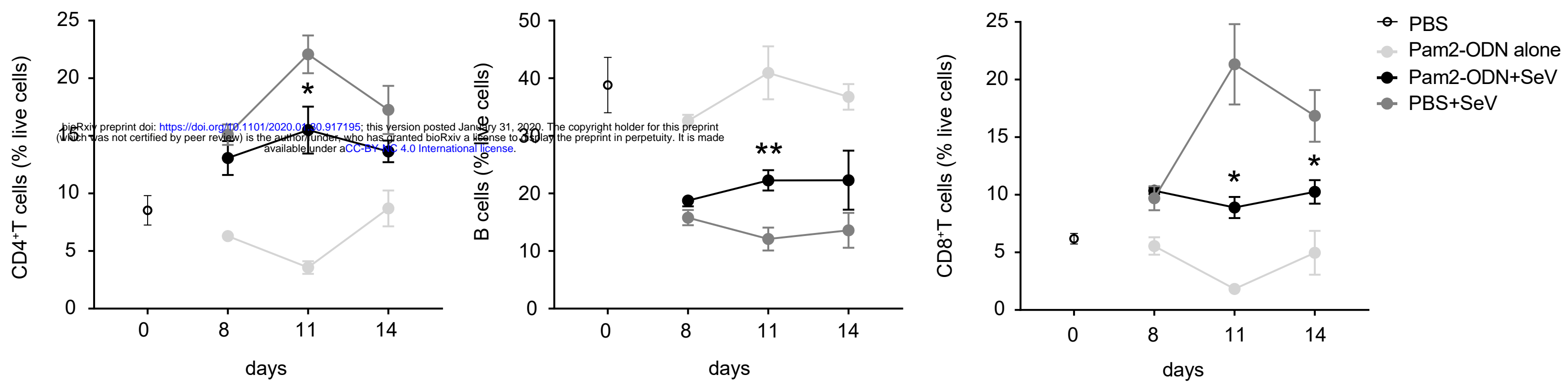Pam2-ODN   −   +
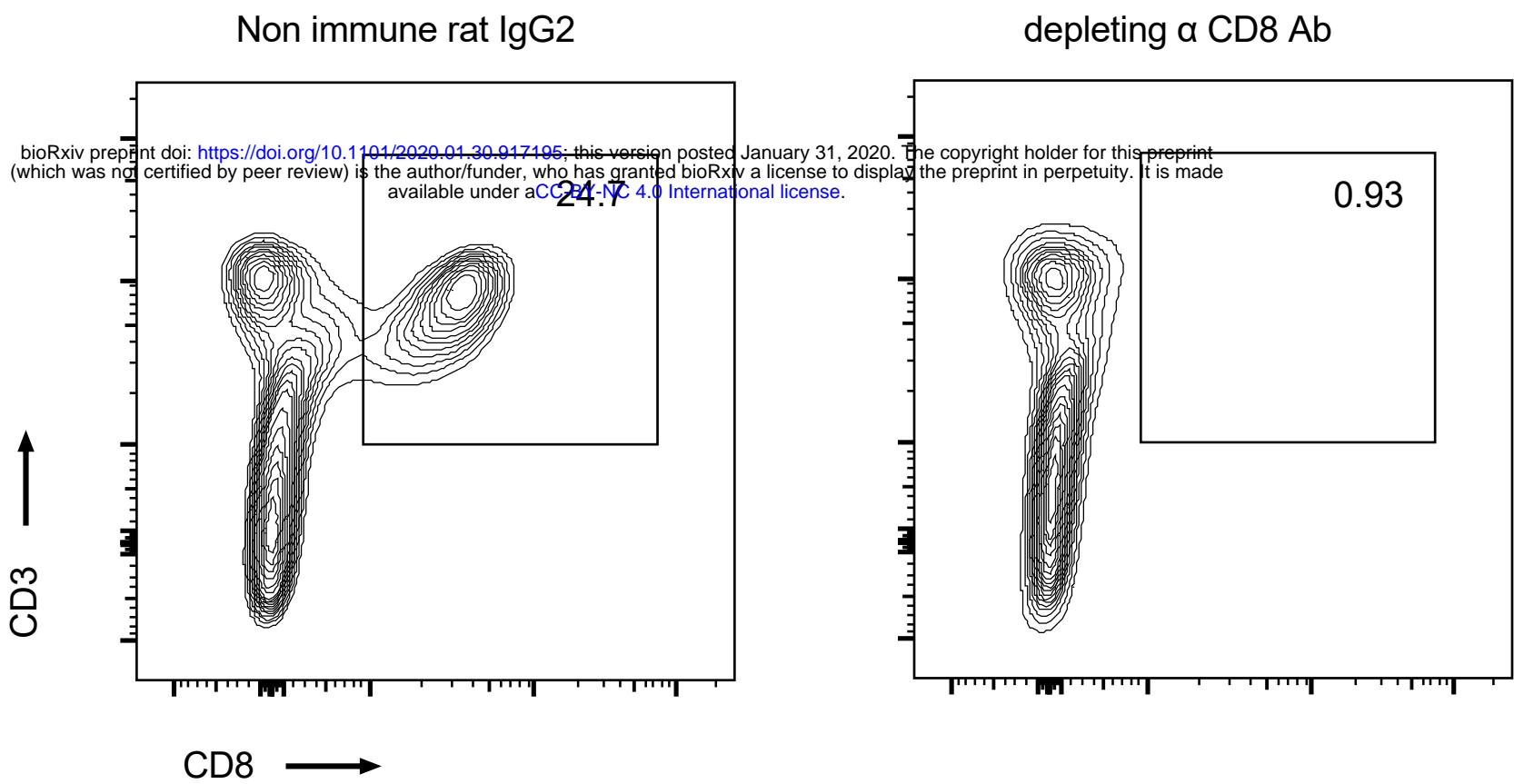SeV   +   +

Pam2-ODN   −   +
SeV   +   +

Wali, Supplementary Figure 1

Wali, Supplementary Figure 2

Wali, Supplementary Figure 3

Non immune rat IgG2

depleting α CD8 Ab

24.7

0.93

CD3

CD8

Wali, Supplementary Figure 4