1   **Mucosal vaccination with CysVac2/Advax induces Th17 lung-resident CD4^{+} T cells and**

2   **interleukin-17-dependant protection against *Mycobacterium tuberculosis* infection**

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Abstract

The development of effective vaccines against bacterial lung infections requires the induction of protective, pathogen-specific immune responses without deleterious inflammation within the pulmonary environment. Here, we made use of a polysaccharide-adjuvanted vaccine approach to elicit resident pulmonary T cells to protect against aerosol *Mycobacterium tuberculosis* infection. Intratracheal administration of *M. tuberculosis* fusion protein CysVac2 and the delta-inulin adjuvant Advax™ (formulated with a TLR9 agonist) provided superior protection against aerosol *M. tuberculosis* infection in mice, compared to parenteral delivery. Surprisingly, removal of the TLR9 agonist did not impact vaccine protection despite a reduction in cytokine-secreting T cell subsets, particularly $CD4^+$ $IFN-\gamma^+IL-2^+TNF^+$ multifunctional T cells. CysVac2/Advax-mediated protection was associated with the induction of lung-resident, antigen-specific memory $CD4^+$ T cells that expressed IL-17 and RORγt, the master transcriptional regulator of Th17 differentiation. IL-17 was identified as a key mediator of vaccine efficacy, with blocking of IL-17 during *M. tuberculosis* challenge reducing phagocyte influx, suppressing priming of pathogen-specific $CD4^+$ T cells in local lymph nodes and ablating vaccine-induced protection. These findings suggest that tuberculosis vaccines such as CysVac2/Advax that are capable of eliciting Th17 lung-resident memory T cells are promising candidates for progression to human trials.

Author summary

Mycobacterium tuberculosis, the causative agent of tuberculosis (TB), kills more individuals each year than any other single pathogen and a more effective vaccine is critical for global control of the disease. TB is an airborne disease, and the only available vaccine, BCG, administered intradermally, is unreliable in protecting against the pulmonary form of the disease. Vaccination in the lung would be a rational way of inducing a local memory immune response to TB, however vaccine platforms would need to deliver antigens to delicate mucosal surfaces without inducing deleterious inflammatory responses. In this report we used pulmonary delivery of TB antigens combined with non-reactogenic polysaccharide adjuvants to induce protection against TB lung infection in mice. The vaccine formulation induced high levels of T cells that resided in the lung and notably expressed the cytokine IL-17. Removal of IL-17 limited the influx of immune cells recruited to the lung and completely ablated protection afforded by the vaccine. This study provides new insights into mechanisms of protection against *M. tuberculosis* and provides a promising candidate to protect against TB in humans.

Introduction

Tuberculosis (TB) remains a major cause of morbidity and mortality worldwide, with 10 million new cases and 1.7 million deaths per year[1]. *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is currently the only licensed vaccine against TB, however its efficacy varies greatly, especially against the adult pulmonary form of the disease[2]. The 2015 WHO End TB Strategy identified the development of a more effective and easily administered vaccine for controlling TB and halting the global epidemic[3]. In recent decades, extensive research has resulted in many new TB vaccine candidate, 14 of which are currently in human vaccine trials and are reviewed in detail elsewhere[4]. Recently, a Phase IIb clinical trial of the fusion protein vaccine M72/AS01$_E$ showed protective efficacy of 50% in *M. tuberculosis*-infected adults after 3 years [5, 6]. Although promising, vaccines with higher efficacy are considered necessary to reduce TB incidence to the targets outlined in the End TB Strategy objectives[3].

One of the major limitations of current vaccination strategies is that the administration route may not be optimal for the induction of immunity at the site of pathogen entry, i.e. the lung. Pulmonary vaccine delivery has been hindered by the fact that most adjuvants are either unable to induce sufficient mucosal immunity or are too toxic to be administered to the lung[7]. However, recent evidence supports the idea that mucosal vaccination may provide superior protection against respiratory *M. tuberculosis* infection over parenteral vaccination. For example, lung-resident CD4$^+$ memory T cells (T$_{RM}$) induced after pulmonary vaccination with a recombinant influenza virus expressing *M. tuberculosis* antigens provided protection in the lung in the absence of circulating memory cells[8]. T$_{RM}$ have also been proposed as the possible mechanism of protection in macaques that demonstrate sterilizing immunity after intravenous vaccination with BCG and subsequent *M. tuberculosis* infection [9]. When administered through the mucosal route, BCG induced increased protection compared to the intradermal immunization, which was linked to lung T$_{RM}$ and Th17 polarization of the CD4$^+$ T cells[10]. Th17 responses have been associated with the influx of neutrophils with

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85   bactericidal activity[11] and increased CD4$^+$ T cell recruitment to the lung after *M. tuberculosis*

86   infection[12]. Vaccines inducing high levels of pulmonary IL-17 have demonstrated efficacy against

87   *M. tuberculosis* in different animal models[13, 14] although balancing the protective and pathogenic

88   roles of IL-17 in the lung is a critical consideration[15].

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90   In this study we sought to determine if the candidate TB vaccine, CysVac2/Advax[16], is effective

91   as a mucosal vaccine to protect against *M. tuberculosis.* CysVac2 is a fusion protein of two *M.*

92   *tuberculosis* antigens; the immunodominant Ag85B and CysD, a component of the sulfur

93   assimilation pathway that is overexpressed in chronic stages of infection. [17] Advax is a particulate

94   polysaccharide adjuvant with a low inflammatory profile that has proven to be safe and a strong

95   inducer of vaccine immunogenicity in humans, thus making it an ideal candidate for mucosal

96   administration[18]'[19]. Notably, it was recently shown to provide safe and effective enhancement of

97   influenza vaccine immunity when administered via the intrapulmonary route in different animal

98   models[20, 21].

99

100   We report here that intrapulmonary administration of CysVac2/Advax induced greater protection in

101   mice than parenterally administered vaccine, with the vaccine promoting the accumulation of antigen-

102   specific, IL-17-secreting CD4$^+$ T$_{RM}$ in the lungs. Furthermore, IL-17 was essential for the protective

103   efficacy afforded by intrapulmonary CysVac2/Advax vaccine, thus defining a crucial role for this

104   cytokine in vaccine-mediated control of TB.

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Results

Pulmonary administration of CysVac2/Advax^{CpG} provides superior protection against *M. tuberculosis* challenge than parenteral vaccination

Previous studies of intramuscular (i.m.) vaccination of mice with CysVac2/Advax^{CpG} demonstrated substantially enhanced systemic CD4$^+$ T cell responses composed of multifunctional Th1 polarized cells, which correlated with protection against aerosol *M. tuberculosis* infection[16]. In this study, we evaluated if delivery to the lung by intratracheal (i.t.) instillation of this vaccine candidate could improve the level of protection induced by this vaccine. Mice were vaccinated by either the i.t. or i.m. routes with CysVac2/Advax^{CpG} 3 times, 2 weeks apart (Fig 1a). When the vaccine-specific T cell response was examined in the blood prior to *M. tuberculosis* challenge, a higher level of circulating polyfunctional CD4$^+$ T cells expressing IFN-γ were present after i.m. vaccination (Fig 1b, S1a Fig), with the most prominent phenotype identified as multi-cytokine secreting CD44$^+$ CD4$^+$ T cells (Fig 1c). After i.t vaccination with CysVac2/Advax^{CpG}, PBMC-derived CD4$^+$ T cell expressing either IL-2, TNF or IL-17 were more prominent when compared to the i.m route (Fig 1b). Both vaccination regimens induced similar proportions of T-bet expression in circulating CD4$^+$ T cells, however i.t. vaccination induced a higher proportion of cells expressing RORγT (Fig 1d).

The pattern of CD4$^+$ T cells immune responses pre-*M. tuberculosis* challenge was compared to that after *M. tuberculosis* infection. In i.m. vaccinated mice, the greatest frequency of CD4$^+$ T cells observed were those secreting IFN-γ or TNF (Fig 1e) and this was dominated by cells with a polyfunctional Th1 responses (IFN-γ$^+$IL-2$^+$TNF$^+$, Fig 1f). I.t. vaccination with CysVac2/Advax^{CpG} resulted in a high frequency of CD4$^+$ T cells secreting either IL-17 or TNF (Fig 1e) or both cytokines (S1b Fig); these T cell subsets were not observed after i.m. vaccination (Fig 1e, S1 Fig). Further, the frequency of CD4$^+$ T cells expressing RORγT was significantly enhanced after i.t. vaccination compared to unvaccinated or i.m. vaccinated mice (Fig 1g). Therefore i.t. vaccination of mice with

132   CysVac2/Advax^{CpG} results in a Th17-polarized T cell response post-*M. tuberculosis* exposure, which

133   was not observed after i.m. immunization.

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135   Considering the differential pattern of immune responses induced by varying the route of

136   administration of CysVac2/Advax^{CpG}, we next determined if this had an impact on protective efficacy.

137   I.t. vaccinated mice challenged with low dose aerosol *M. tuberculosis*, demonstrated significantly

138   enhanced lung protection when compared to i.m.-vaccinated or unvaccinated mice (Fig 1h). A similar

139   result was observed in the spleen, suggesting that i.t. vaccination might improve protection against

140   disseminated infection (Fig 1i). Taken together, these results demonstrate that pulmonary vaccination

141   with CysVac2/Advax^{CpG} induces superior protection compared to i.m. vaccination and this is

142   associated with an enhanced generation of Th17 cells in the circulation and in the lung.

143

144   **CpG is dispensable for protection generated by pulmonary vaccination with CysVac2/Advax**

145   CpG oligonucleotides are TLR9 agonists that help drive Th1 immune responses[22]. While the CpG

146   component was shown to be important to the protection obtained after CysVac2 i.m. immunization,

147   we were interested whether a simplified formulation of Advax without the CpG component would

148   still generate protective pulmonary immunity. CysVac2/Advax or CysVac2/Advax^{CpG} were delivered

149   by the i.t. route, and the mice challenged with aerosol *M. tuberculosis*. Both vaccines resulted in the

150   generation of IL-17-producing CD4$^+$ T cells following antigen restimulation *ex vivo* (Fig 2A),

151   however all inflammatory cytokines (IFN-γ, IL-17, TNF) were reduced in Advax-immunized as

152   compared to Advax^{CpG}-vaccinated mice (Fig 2a). Strikingly, the removal of the CpG component

153   resulted in the loss of multifunctional CD4$^+$ T cells with a triple cytokine-secreting profile (IFN-γ$^+$IL-

154   2$^+$TNF$^+$) (Fig 2b). However, both vaccinated groups displayed equivalent expression of the

155   transcription factors T-bet or RORγT (Fig 2c) and similar protection against *M. tuberculosis* in the

156   lungs (Fig 2c) and spleen (Fig 2d). This indicates that CysVac2/Advax is sufficient for protection and

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157   this protection does not correlate with the presence of multifunctional T cells secreting high levels of

158   inflammatory cytokines.

159

160   **Intrapulmonary CysVac2/Advax generates lung-resident, antigen-specific CD4$^+$ T cells**

161   To more precisely define the vaccine-specific responses after immunization with CysVac2/Advax,

162   Ag85B:I-A^b tetramer staining was employed to identify CD4$^+$ T cells specific for the p25 epitope of

163   Ag85B, an antigenic component of CysVac2[17]. Ag85B tetramer-positive (Ag85Btet$^+$) cells in the

164   lungs were only detected after i.t. delivery of CysVac2/Advax and not after CysVac2 antigen alone,

165   confirming the critical role of Advax in inducing antigen-specific T cell expansion (Fig 3a).

166   Ag85Btet$^+$ cells were present in significantly greater numbers in CysVac2/Advax vaccinated samples

167   at all timepoints examined, although numbers contracted by 8 weeks post-vaccination (Fig 3b).

168   CysVac2/Advax-vaccinated mice showed enrichment of lung parenchymal-residing (intravascular

169   negative IV$^-$) CD4$^+$ T cells, which expressed CD69 and CD44 with low level of expression of the

170   lymphoid   homing   receptor,   L-selectin   (CD62L)   (Fig   3d).   This   population   of

171   CD4$^+$CD44^{hi}CD62L^{low}CD69$^+$ IV$^-$ were defined as T$_{RM}$-like cells[23] and were significantly greater

172   at all times points post-vaccination in CysVac2/Advax-vaccinated mice, comprising approximately

173   25% of total CD4$^+$ T cells in the lung following vaccination (Fig 3c). The majority of Ag85B-tet$^+$

174   cells detected at 8 weeks post vaccination showed a T$_{RM}$-like phenotype and were present within the

175   parenchyma of the lung (Fig 3e). Along with T$_{RM}$ markers, this subset expressed high levels of the

176   integrin CD11a and the cell surface receptor PD-1, with low levels of expression of CD103 and

177   KLRG-1 (Fig 3e). Taken together, these data indicate that CysVac2/Advax vaccination induces a

178   population of antigen-specific CD4$^+$ T cells in the lung with a T$_{RM}$ phenotype, and these are detectable

179   through 8 weeks post vaccination.

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181   We further characterized the ability of the CD4$^+$ T cells generated in the lung to produce cytokines

182   in response to re-stimulation with vaccine antigen at time points after vaccination (pre-challenge) as

183   well as at 4 weeks post-challenge with *M. tuberculosis*. Intratracheal CysVac2/Advax vaccination

184   induced a distinct cytokine profile that was relatively consistent across all time points prior to *M.*

185   *tuberculosis* infection. While a marginal increase in IFN-γ production was observed at 4 weeks post

186   vaccination, this was not apparent at any other time point (Fig 4a). Indeed, following infection with

187   *M. tuberculosis*, a lower percentage of IFN-γ-producing CD4+ T cells was present in CysVac2/Advax

188   vaccinated lung samples compared with samples from unvaccinated animals. By contrast,

189   percentages of of IL-2, IL-17 or TNF cytokine-producing cells were markedly increased after i.t.

190   vaccination with CysVac2/Advax compared to unvaccinated or CysVac2 vaccinated mice up to 8

191   weeks post-vaccination (Fig 4b, 4c, 4d), however only IL-17 remained elevated post-challenge (Fig

192   4b). Further analysis revealed that the major source of IL-17 originated from CD45 IV-CD4+ T cells

193   that expressed RORγT (Fig 4e). Furthermore, the Ag85Btet+ population was highly enriched in

194   RORγT+ CD45 IV-IL-17+CD4+ T cells (Fig 4f). Overall, these data suggest that pulmonary

195   vaccination with CysVac2/Advax promotes increased single and multifunctional cytokine producing

196   T cell populations both before and after aerosol challenge with *M. tuberculosis*, which is characterized

197   by the development of a tissue-resident Th17-type response.

198

199   **IL-17-mediated protection correlates with early recruitment of phagocytic cells and enhanced**

200   **priming of pathogen-specific CD4+ T cells**

201   Given the marked Th17 polarization of the CD4+ T cell response to pulmonary immunization with

202   CysVac2/Advax, we next determined the impact of neutralizing IL-17 at the time of *M. tuberculosis*

203   challenge (Fig 5a). Treatment with anti-IL-17 mAb did not affect the capacity of CD4+ T cells to

204   respond to infection, as the frequency of cytokine-producing CD4+ T cells was not altered between

205   mice treated with anti-IL-17 or isotype control mAb (Fig 5b). However, anti-IL-17 treatment had a

206   detrimental effect on control of bacterial infection in the lung of CysVac2/Advax immunised mice;

207   bacteria numbers in anti-IL-17 treated mice were similar to unvaccinated mice, while immunised

208   mice treated with isotype control mAb remained protected against infection (Fig 5c).

209 We also studied the impact of IL-17 blocking on lung cell subsets after *M. tuberculosis* infection

210 using flow cytometry phenotyping combined with an unsupervised visual implementation of t-

211 distributed stochastic neighbour embedding (tSNE) analysis. The generated tSNE plot was calculated

212 with 12 parameters and 10 clusters obtained using unsupervised analysis were subsequently assigned

213 to a specific cell population (Fig 5d), according to expression level of each marker and previously

214 described phenotypes (S3Fig). This analysis revealed that the percentage of neutrophils in the lung

215 were elevated in vaccinated mice treated with control mAb, however they returned to the level of

216 unvaccinted animals after treatment with the anti-IL-17 mAb (Fig 5e). A similar pattern was observed

217 for monocytes and interstitial macrophages, however no differences were observed for other

218 phagocytic populations in the lung, such as alveolar macrophages (Fig 5e).

219

220 We next investigated the effect of blocking IL-17 on the priming and proliferation of $CD4^+$ T cells.

221 To do this, we examined $CD4^+$ T cells primed by the vaccination ($Ag85B$-tet^+) and compared to T

222 cells responding specifically to *M. tuberculosis* ($ESAT6$-tet^+). In the lung we observed a greater

223 proportion of $Ag85B$-tet^+ cells in vaccinated mice compared to unvaccinated mice, however anti-IL-

224 17 treatment did not significantly alter the numbers of proliferating $CD4^+$ T cells (S4a, b, and c Fig)

225 or the numbers of $Ag85B$-tet^+ or $ESAT6$-tet^+ cells (S4d, e and f Fig). However, in the mLN of

226 vaccinated mice a distinct vaccine-induced population of proliferating $CD4^+$ T cells expressing

227 RORγT was distinguishable (Fig 6a). IL-17 blocking resulted in reduced proliferation of total $CD4^+$

228 T cells in the mLN of vaccinated mice (Fig 6b) including the vaccine-primed $RORγT^+$ cells (Fig 6c).

229 When the frequency of the vaccine-primed $CD4^+$ T cells ($Ag85B$-tet^+) were compared to those primed

230 only by *M. tuberculosis* infection ($ESAT6$-tet^+) (Fig 6d), $Ag85B$-tet^+ cells were significantly higher

231 in vaccinated mice compared to the unvaccinated group, however anti-IL-17 treatment did not affect

232 the numbers of $Ag85B$-tet^+ cells (Fig 6e). By contrast, the numbers of $ESAT6$-tet^+ positive $CD4^+$ T

233 cells were reduced after IL-17 blocking in the mLNs (Fig 6f). Overall, neutralizing of IL-17 reduced

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234   phagocytic cells in the lung of immunized animals and the numbers of pathogen-specific CD4$^+$ T

235   cells in the draining LNs, which correlated with a loss of vaccine-mediated protection.

236

237   **Discussion**

238   The respiratory tract is the preferred port of entry of *M. tuberculosis*, with the complexity of the

239   immunological environment in the lung potentially contributing to suboptimal pathogen responses.

240   This may be particularly detrimental in the case of *M. tuberculosis* infection, where priming and

241   recruitment of effector T lymphocytes to the lungs is delayed, allowing unchecked growth of the

242   organism[24, 25]. For this reason, mucosal vaccination has been of interest in the field of TB vaccines,

243   with pulmonary delivery of BCG[14, 26], live recombinant viruses[8, 27, 28], or protein/adjuvants[29,

244   30] resulting in protective immune responses. When administered to the respiratory mucosa, highly

245   inflammatory adjuvanted vaccines may induce protective immunity, but this is often accompanied by

246   excessive inflammation, mucus accumulation and eosinophilia[31]. Thus, there is a need for

247   adjuvants that can induce protective lung immunity in the absence of deleterious inflammation and

248   pathology. We found that i.t. delivery of CysVac2/Advax^{CpG} was significantly more protective than

249   i.m. vaccination against challenge with *M. tuberculosis* (Fig 1). Strikingly i.t. vaccination, despite

250   being more protective, did not induce appreciable numbers of multifunctional CD4$^+$ T cells (IFN-

251   γ$^+$IL-2$^+$TNF$^+$; Fig 1) and even more surprisingly removal of CpG from the formulation did not reduce

252   vaccine protection, despite a loss of multifunctional CD4$^+$ T cell generation (Fig 2). Induction of

253   multifunctional T cell responses has been used as a key criteria for vaccine progression to human

254   trials, however recent evidence in both mice and humans indicates that the generation of IFN-γ

255   secreting T cells does not necessarily correlate with protection[32]. Indeed, in our data IFN-γ was the

256   only cytokine analyzed pre- and post-challenge that did not correlate with the protective effect of the

257   CysVac2/Advax vaccine (Fig 4), an important finding for the selection of vaccines for progression to

258   human trials.

259

260   We observed that pulmonary vaccination with CysVac2 vaccination induced a lung resident CD4$^+$

261   population that expressed markers of T$_{RM}$-like cells (Fig 3). We have previously shown that a

262   recombinant influenza vaccine conferred protection against *M. tuberculosis* in the absence of

263   circulating memory T cells, suggesting an important role for *M. tuberculosis*-specific T_{RM}[8]. In the

264   current study, detailed phenotypic analysis of CysVac2/Advax-induced, antigen-specific CD4$^+$ T_{RM}-

265   like cells showed that in addition to well-characterized markers of tissue-resident memory cells

266   (CD69$^+$ CD44^{hi} CD62L^{low} CD45 IV$^-$), this population displayed high levels of CD11a with minimal

267   expression of CD103 (Fig 3). This low level of CD103 expression contrasts with the increased

268   expression of CD103 on CD8 T_{RM}, where this integrin is thought to be essential in the retention of

269   these cells within tissue[33]. Antigen-specific T_{RM} cells induced following CysVac2/Advax

270   vaccination also displayed a dominant PD-1$^+$ KLRG-1$^-$ phenotype (Fig 3). During *M. tuberculosis*

271   infection, PD-1 expression indicates an earlier stage of CD4$^+$ T cell differentiation that is associated

272   with a higher proliferative capacity, while KLRG-1$^+$ cells are more terminally differentiated and

273   produce greater levels of cytokines[34]. IL-2-secreting KLRG-1$^-$ T cells induced by subunit booster

274   vaccination are associated with protection against chronic *M. tuberculosis*, owing to maintenance of

275   their proliferative capacity[35], and circulating KLRG-1$^-$ CD4$^+$ T cells induced by subcutaneous

276   H56/CAF01 vaccination display the ability to home to the lungs[36]. Our findings confirmed an

277   association between vaccine-induced, PD-1$^+$KLRG-1$^-$ CD4$^+$ T cells that reside within the lung

278   parenchyma and protection against *M. tuberculosis*. It is possible these cells possess greater effector

279   functions and proliferative capacity than their KLRG-1$^+$ counterparts and are therefore capable of

280   enhanced protection against *M. tuberculosis* infection.

281

282   Lung-resident CD4$^+$ T_{RM} were found to be an important source of IL-17 induced after

283   Advax:CysVac2 i.t. delivery, and this was not observed after i.m. delivery of the vaccine. This is in

284   agreement with other studies showing that IL-17 is strongly induced following mucosal vaccination

285   and correlates with protection against *M. tuberculosis* infection[37, 38], yet the mechanism by which

286   this protection is specifically mediated is not well defined. Blocking of IL-17 during *M. tuberculosis*

287   challenge in CysVac2/Advax-vaccinated mice resulted in a complete loss of vaccine-induced

288   protection and correlated with reduced recruitment of lung phagocytic cells such as neutrophils and

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289 macrophages, as well as a reduced priming of *M. tuberculosis*-specific T cells in the mLN (Fig 5, 6).

290 Thus we can propose a mechanism (Fig 7) whereby IL-17 production by vaccine-specific CD4$^+$ T$_{RM}$

291 facilitates neutrophil and Monocytes/macrophages recruitment[11], and promotes the activation and

292 proliferation of protective, pathogen-specific CD4$^+$ T cells in the mLN [39], [40],[41]. Interstitial

293 macrophages recruitment might be beneficial because these populations appear to possess significant

294 antimycobacterial activity, as opposed to alveolar macrophages [42]. Previous work in a different

295 infectious model has additionally demonstrated that IL-17 can mediates the recruitment of neutrophils

296 with anti-bacterial potential[11]. Our observations differs to previous reports using the ID93+GLA-

297 SE mucosal TB vaccine, which resulted in a Th17-dominated, tissue-resident response, but did not

298 lead to improved protection when compared to parenteral immunization[43]. This may indicate that

299 different adjuvants may induce diverse T$_{RM}$ populations, and the capacity of Advax to direct a

300 predominant, vaccine-specific T$_{RM}$ response in the lung may be a key determinant in the protective

301 efficacy of CysVac2/Advax vaccine.

302

303 Harnessing the protective role of IL-17 against pathogens needs to be balanced with potential tissue

304 damage and pathology associated with excess cytokine levels[15]. Thus, adjuvants stimulating

305 excessive levels of IL-17 are unlikely to be suitable for human lung administration, as observed for a

306 candidate Sporotrichosis vaccine[44]. When CpG was removed from the CysVac2/Advax vaccine,

307 the frequency of IL-17 secreting T cells in the lung was reduced, yet protection against *M.*

308 *tuberculosis* challenge was not affected (Fig 2, S2 Fig). This suggests that a threshold of IL-17-

309 secreting T cells may exist for vaccine-induced protection against *M. tuberculosis* and selecting the

310 most 'immunogenic' vaccines based on the greatest level of effector responses may not be the best

311 strategy for identifying an optimal *M. tuberculosis* vaccine formulation.

312

313 In conclusion, this report demonstrates that Advax-adjuvanted vaccines can be safely delivered to the

314 lung to provide significant protection against aerosol *M. tuberculosis* infection. The protective effect

315   of the vaccine was associated with targeted expansion of lung-resident T_{RM} and was dependent on

316   IL-17 recruitment of phagocytic cells to the lung and enhanced priming of T cells in the mLN. As

317   Advax-containing vaccines have proven safe and immunogenic in human trials against viral infection

318   and allergy[19, 45, 46] and are safe and effective in pre-clinical trials as inhaled formulations[21],

319   CysVac2/Advax is a promising candidate for assessment of safety, immunogenicity and efficacy as

320   a pulmonary vaccine in human subjects.

Materials and Methods

Ethics statement

Female C57BL/6 (6-8 weeks of age) were purchased from Australian BioResources (NSW, Australia), and housed at the Centenary Institute animal facility (Sydney, Australia) in specific pathogen-free conditions. All mouse work was performed according to ethical guidelines as set out by the University of Sydney Animal Ethics Committee. All experiments within this manuscript were approved under protocol number 2017/011. University of Sydney Animal Ethics Committee guidelines adhere to the Australian Code for the Care and Use of Animals for Scientific Purposes (2013) as set out by the National Health and Medical Research Council of Australia.

Bacterial strains

M. tuberculosis H37Rv (BEI Resources, USA) and BCG Pasteur were grown at 37° C in Middlebrook 7H9 medium (Becton Dickinson, BD) supplemented with 0.5 % glycerol, 0.02 % Tyloxapol, and 10 % albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (BD) supplemented with oleic acid–ADC.

Mouse immunization, treatments and infection

CysVac2 fusion protein (Ag85B-CysD) was recombinantly expressed in *E. coli*, purified from inclusions bodies by ChinaPeptides (Shanghai, China), and refolded in Tris-buffer. Advax (delta-inulin, 50 mg/ml) and Advax^{CpG} (delta-inulin plus CpG, 50 mg/ml and 500 μg/ml, respectively) were provided by Vaxine Pty Ltd (Adelaide, Australia). Mice were anaesthetized by intraperitoneal (i.p.) injection of Ketamine/Xylazine (80/100mg/kg mouse) and then vaccinated with 1 mg of Advax or Advax^{CpG} and 3 μg of CysVac2 in a final volume of 50 μL PBS via i.m. route, using an insulin syringe (BD), or via the i.t. route, using PennCentury Microsprayer Aerosoliser (PennCentury, PA, USA). Three μg of CysVac2 alone or sterile PBS were administered as controls where appropriate. Mice were immunized subcutaneously with 5x10^5 BCG for protection experiments.

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348   For neutralization of IL-17, mice were injected i.p. with 250 μg of anti-IL-17A (clone TC11-18H10.1,

349   Biolegend) or isotype control (clone RTK2071, Biolegend) one day prior to *M. tuberculosis* infection

350   and then every three days for three weeks.

351

352   For *M. tuberculosis* challenge experiments, six or 8 weeks after the last vaccination mice were

353   infected with *M. tuberculosis* H37Rv via the aerosol route using a Middlebrook airborne infection

354   apparatus (Glas-Col, IN, USA) with an infective dose of approximately 100 viable bacilli. Three or

355   4 weeks later, the lungs and spleen were harvested, homogenized and plated after serial dilution on

356   supplemented Middlebrook 7H11 agar plates. Colonies forming units (CFU) were determined 3

357   weeks later and expressed as Log_{10} CFU.

358

359   For intravascular staining of leucocytes, three minutes before euthanasia, mice were i.v. injected with

360   200 μL biotin-conjugated anti-CD45 mAb in PBS (15 μg/mL, Biolegend, clone 104) into the lateral

361   tail vein. Detection of biotin was performed with APC-Cy7-conjugated streptavidin (BioLegend).

362

363   **Cell isolation, peptide stimulations and flow cytometry**

364   PBMCs were isolated from whole blood as previously described[17]. Single cell suspensions were

365   prepared from the lung as previously described[17]. PE-conjugated $Ag85B_{240-254}$:I-A^b tetramer and

366   APC-conjugated $ESAT6_{1-20}$:I-A^b tetramer were provided by the NIH Tetramer Core Facility. For

367   staining, cells were incubated with tetramers at 37 °C for 1 hour. Cells were stained using the marker-

368   specific fluorochrome-labeled mAbs indicated in S1 Table. To assess antigen-specific cytokine

369   induction by T cells, PBMCs or single-cell suspensions from the lung were stimulated for 4 hours

370   with CysVac2 (5 μg/mL) and then supplemented with brefeldin A (10 μg/mL) for further 10-12 hours.

371   Cells were surface stained with Fixable Blue Dead Cell Stain (Life Technologies) and the marker-

372   specific fluorochrome-labeled antibodies indicated in Supplementary Table. Cells were then fixed

373   and permeabilized using the BD Cytofix/Cytoperm™ kit according to the manufacturer's protocol.

374   When required, intracellular staining was performed using mAbs against the specific cytokines (S1

375   Table). Samples were acquired on a BD LSR-Fortessa (BD), and analyzed using FlowJo™ analysis

376   software (Treestar, USA). A Boolean combination of gates was used to calculate the frequency of

377   single-, double- and triple-positive $CD4^+$ T cell subsets. tSNE was run using default FlowJo

378   parameters (iterations = 1000, perplexity = 30). Samples were randomly downsampled to 2000 events

379   per sample and analysis was run on equal numbers of events per sample using FlowJo tSNE plugin.

380

381   **Statistical analysis**

382   Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, USA). The

383   significance of differences between experimental groups was evaluated by one-way analysis of

384   variance (ANOVA), with pairwise comparison of multi-grouped data sets achieved using the Tukey

385   post-hoc test. Differences are considered statistically different when $p \leq 0.01$.

386

395

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413   **Conflict of Interest Disclosure:** NP is the research director for Vaxine P/L. All authors attest they

414   meet the criteria for authorship.

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521   PMCID: PMCPMC6490884 following competing interests: HMcS is a Jenner Institute Investigator

522   and a Wellcome Trust Senior Clinical Research Fellow. ZM was a NIHR BRC Clinical Research

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598

599   **Figure Legends**

600

601   **Figure 1. Pulmonary vaccination with CysVac2/Advax^{CpG} demonstrates improved protection**

602   **against *M. tuberculosis* infection compared to parenteral administration.** C57BL/6 mice (n=5-6)

603   were vaccinated by either the i.m. or i.t. route with CysVac2(CV2)/Advax^{CpG} (3 times, 2 weeks apart).

604   One week after last vaccination mice were bled for vaccine immunogenicity assessment. Six weeks

605   after last immunization mice were challenged with H37Rv by aerosol (~100 CFU) and four weeks

606   later culled to enumerate bacterial burden and T cell phenotype in the lung (a). PBMCs from tail

607   blood of vaccinated mice (b, c, d) or cells from lung of infected mice (e, f, g) were restimulated *ex*

608   *vivo* with CysVac2, and the production cytokines (IFN-γ, IL-2, IL-17, TNF), or transcription factors

609   (TF; T-bet, RORγT) by CD4$^+$ T cells was determined by flow cytometry. Data are represented as the

610   percentage of cytokine-producing or transcription factor-positive CD4$^+$ T cells ± SEM. Bacterial load

611   was assessed in the lungs (h) and in the spleen (i) and presented as Log$_{10}$ of the mean CFU ± SEM.

612   Data are pooled from 3 independent experiments. Significance of differences between the groups was

613   determined by ANOVA (*p<0.05; **p<0.01; ***p<0.001).

614

615   **Figure 2. CpG is dispensable for protective immunity induced by CysVac2/Advax.** C57BL/6

616   mice (n=5-6) were vaccinated by the i.t. route with CysVac2/Advax^{CpG} or CysVac2/Advax (3 times,

617   2 weeks apart). Six weeks after last immunization mice were challenged with *M. tuberculosis* H37Rv

618   by aerosol (~100 CFU) and four weeks later culled to enumerate bacterial burden and T cell

619   phenotype in the lung. Cells from lung of infected mice were restimulated *ex vivo* with CysVac2 and

620   the production of cytokines (IFN-γ, IL-2, IL-17, TNF; panel a and b) or transcription factors (T-bet,

621   RORγT; panel c) by CD4$^+$ T cells was determined by flow cytometry. Data are represented as the

622   percentage of cytokine-producing CD4$^+$ T cells ± SEM. Bacterial load was assessed in the lungs (d)

623   and in the spleen (e) and presented as Log$_{10}$ of the mean CFU ± SEM. Data are pooled from 2

28

624 independent experiments. Significance of differences between the groups was determined by

625 ANOVA (*p<0.05; **p<0.01; ***p<0.001).

626

627 **Figure 3. Pulmonary vaccination with CysVac2/Advax induces Ag-specific persistent local**

628 **resident CD4$^+$ T cells.** C57BL/6 mice (n=3-4) were vaccinated by the i.t. route with CysVac2/Advax

629 or CysVac2 (3 times, 2 weeks apart). At weeks 2, 4, or 8 after final immunization, lung cells were

630 processed for Ag85B:I-A^b tetramer staining (a, Representative dot plot from 8 weeks post-

631 immunization). Number of Ag85B:I-A^b tetramer positive cells in the lung over time are shown in (b).

632 Also shown are the percentage of total CD4$^+$ T cells expressing phenotypic makers markers

633 associated with TRMs (CD45 IV$^-$, CD11a$^+$, CD69$^+$, CD44$^+$, PD1$^+$ KLRG$^-$, c) and representative dot

634 plots of TRMs markers on total lung CD4$^+$ T cells (d) or in Ag85B:I-A^{b+} cells (e) at 8 weeks after

635 last vaccination. Data are representative of 2 independent experiments. Significance of differences

636 between the groups was determined by ANOVA (*p<0.05; **p<0.01; ***p<0.001).

637

638 **Figure 4. Persistent CysVac2-specific IL-17 production by lung-resident CD4$^+$ T cells after**

639 **pulmonary vaccination with CysVac2/Advax.** C57BL/6 mice (n=4) were vaccinated by the i.t.

640 route with CysVac2/Advax or CysVac2 protein alone (3 times, 2 weeks apart). Eight weeks after the

641 last immunization mice were challenged with *M. tuberculosis* H37Rv by aerosol (~100 CFU). At 2,

642 4, or 8 weeks after last immunization (solid bars) , and at 4 weeks after infection (striped bars), lung

643 cells were restimulated *ex vivo* with CysVac2 and the production of IFN-γ (a), IL-17 (b), IL-2 (c) or

644 TNF (d) by CD4$^+$ T cells determined by flow cytometry. Representative dot plots of co-expression

645 of CD45 IV or RORγT with IL-17 by total CD4$^+$ T cells (e) or Ag85B:I-A^b tetramer positive cells (f)

646 at 8 weeks after last vaccination. Data are represented as the percentage of cytokine-producing CD4$^+$

647 T cells ± SEM and is representative of 2 independent experiments. Significance of differences

648 between the groups was determined by ANOVA (*p<0.05; **p<0.01; ***p<0.001).

649

650 **Figure 5. Protection afforded by pulmonary CysVac2/Advax against aerosol *M. tuberculosis* is**

651 **dependent on IL-17 and correlates with lung phagocytic cells recruitment.** C57BL/6 mice (n=6)

652 were vaccinated by the i.t. route with CysVac2/Advax (3 times, 2 weeks apart) and at 8 weeks after

653 last immunization mice were challenged with *M. tuberculosis* H37Rv by aerosol (~100 CFU). One

654 day before aerosol, mice were treated i.p. with anti-IL1-7 or an isotype control mAb (twice weekly

655 for 3 weeks) (a). Cells from the lungs of infected mice were restimulated *ex vivo* with CysVac2 and

656 cytokines secretion (IFN-γ, IL-2, IL-17, TNF) determined (b). Bacterial load was assessed in the

657 lungs and is presented as Log_{10} of the mean CFU ± SEM (c). Representative tSNE dimension 1 and

658 2 plots of the total live cells in the lung (d). Bar graphs show the percentage of identified lung cells

659 subsets (e). Data are pooled of 2 independent experiments. Significance of differences between the

660 groups was determined by ANOVA (*p<0.05; **p<0.01; ***p<0.001).

661

662 **Figure 6. Blocking IL-17 during *M. tuberculosis* infection impairs the proliferation of pathogen-**

663 **specific CD4+ T cells in the mediastinal lymph nodes.** C57BL/6 mice (n=6) were vaccinated with

664 CysVac2/Advax and treated i.p. with anti-IL-17 mAb, as described in Figure 5. Representative dot

665 plot of the expression of Ki67 and RORγT on CD4+ T cells (a). Bar graphs show numbers of total (b)

666 and RORγT+ (c) proliferating CD4+ T cells enumerated in the mLN. Representative dot plots show

667 CD44 and either Ag85B:I-A^b or ESAT6:I-A^b staining on CD4+ T cells in the mLN (d), with total

668 number ± SEM of Ag85B:I-A^{b+} (e) and ESAT6:I-A^{b+} (f) CD4+ T cells in the mLN. Data are pooled

669 of 2 independent experiments. Significance of differences between the groups was determined by

670 ANOVA (*p<0.05; **p< 0.01).

671

672 **Figure 7. Proposed mechanism of immunity generated by pulmonary vaccination with**

673 **CysVac2/Advax against *M. tuberculosis* infection.** Pulmonary vaccination of CysVac2/Advax

674 induces the development of a Th17 T_{RM} population of antigen-specific CD4+ T cells in the lung.

675 Following aerosol *M. tuberculosis* infection, T cell IL-17 drives neutrophil and macrophage

30

676   recruitment to the lung. This early response may be responsible for increased activation or trafficking

677   of antigen presenting cells to the mLN, which in turn promotes the priming and proliferation of

678   pathogen-specific CD4$^+$ T cells in mLN that migrate to the lung and contribute to protection against

679   *M. tuberculosis* infection.

680

681   **S1 Figure. Comparative analysis of multifunctional CD4$^+$ T cell subsets before and after *M.***

682   ***tuberculosis* infection of mice vaccinated with CysVac2/Advax^{CpG} either via pulmonary or**

683   **parenteral route.**

684   C57BL/6 mice (n=5-6) were vaccinated by either the i.m. or i.t. route with CysVac2 (CV2)/Advax^{CpG}

685   (3 times, 2 weeks apart). One week after last vaccination mice were bled for vaccine immunogenicity

686   assessment. Six weeks after last immunization mice were challenged with H37Rv by aerosol (~100

687   CFU) and four weeks later culled to enumerate bacterial burden and T cell phenotype in the lung.

688   PBMCs (one week after last vaccination, panel a) and lung cells 4-weeks p.i. (panel b) were

689   restimulated with CysVac2 fusion protein, and analysed for intracellular expression of IFN-γ, IL-2,

690   IL-17, and TNF by flow cytometry. Boolean gating analysis was performed to identify subsets of

691   CD4$^+$ T cells expressing different combinations of these cytokines. Data is representative of 2

692   independent experiments. Significance difference between the groups was determined by ANOVA

693   (*$p < 0.05$; **$p < 0.01$).

694

695   **S2 Figure. Comparative analysis of lung multifunctional CD4$^+$ T cell subsets after *M.***

696   ***tuberculosis* infection of mice vaccinated via pulmonary route with either CysVac2/Advax or**

697   **CysVac2/Advax^{CpG}.**

698   C57BL/6 mice (n=5-6) were vaccinated by i.t. route with either CysVac2 (CV2)/Advax or CysVac2

699   (CV2)/Advax^{CpG} (3 times, 2 weeks apart). Six weeks after last immunization mice were challenged

700   with H37Rv by aerosol (~100 CFU), and four weeks later culled to enumerate bacterial burden and

701   T cell phenotype in the lung. Lung cells 4 weeks p.i. were restimulated with CysVac2 fusion protein,

31

702 and analysed for intracellular expression of IFN-γ, IL-2, IL-17, and TNF by flow cytometry. Boolean

703 gating analysis was performed to identify subsets of CD4$^+$ T cells expressing different combinations

704 of these cytokines. Data is representative of 2 independent experiments. Significance difference

705 between the groups was determined by ANOVA (*$p < 0.05$; **$p < 0.01$).

706

707 **S3 Figure. tSNE expression of markers in the lung of mice after *M. tuberculosis* infection and**

708 **anti-IL-17 mAb treatment.** Example of tSNE dimension 1 and 2 plots of the lung compartment

709 show relative expression intensity of each indicated phenotypic marker. tSNE heat maps show

710 fluorescent intensity of each marker for each event. Scales on the heat maps are individually generated

711 for each surface marker from low to high expression. (Ashhurst, T. M. 2017, tSNEplots v1.3. GitHub

712 repository).

713

714 **S4 Figure. Effects of blocking IL-17 during *M. tuberculosis* infection on CD4$^+$ T cells**

715 **proliferation in the lung.**

716 C57BL/6 mice (n=5-6) were vaccinated i.t. with CysVac2/Advax and treated i.p. with anti-IL-17

717 mAb, as described in Figure 5. Representative dot plot of the expression of Ki67 and RORγT on

718 CD4$^+$ T cells from the lung (a). Bar graphs showing numbers of total (b) and RorγT$^+$ (c) proliferating

719 CD4$^+$ T cells enumerated in the lung. Representative dot plots show CD44 and either Ag85B:I-Ab or

720 ESAT6:I-Ab staining on CD4$^+$ T cells in the lung (d), with total number ± SEM of Ag85B:I-Ab$^+$ (e)

721 and ESAT6:I-Ab$^+$ (f) CD4$^+$ T cells in the lung. Data are pooled of 2 independent experiments.

722 Significance of differences between the groups was determined by ANOVA (*$p<0.05$; ***$p<0.001$;

723 NS=not significant).
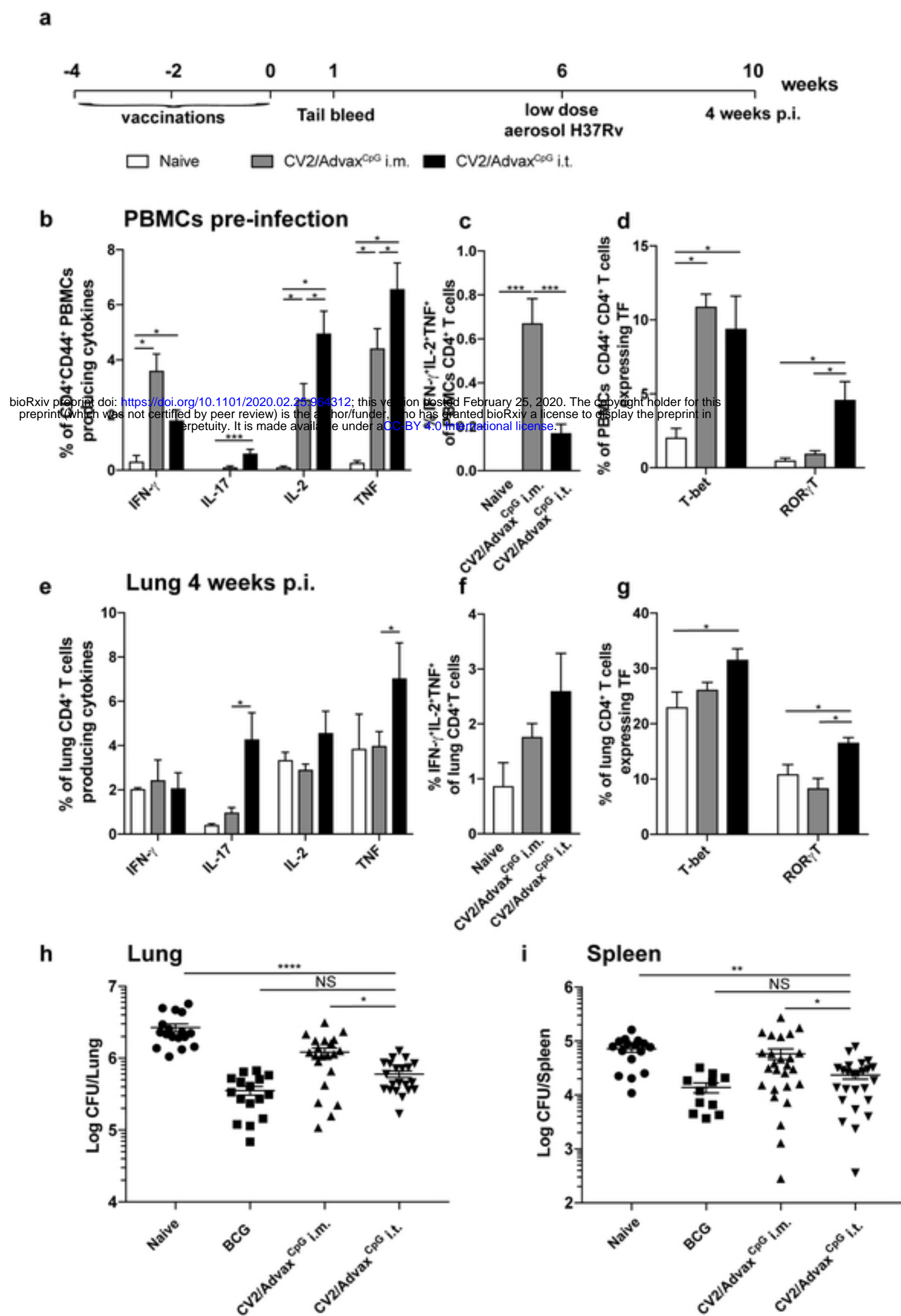
724

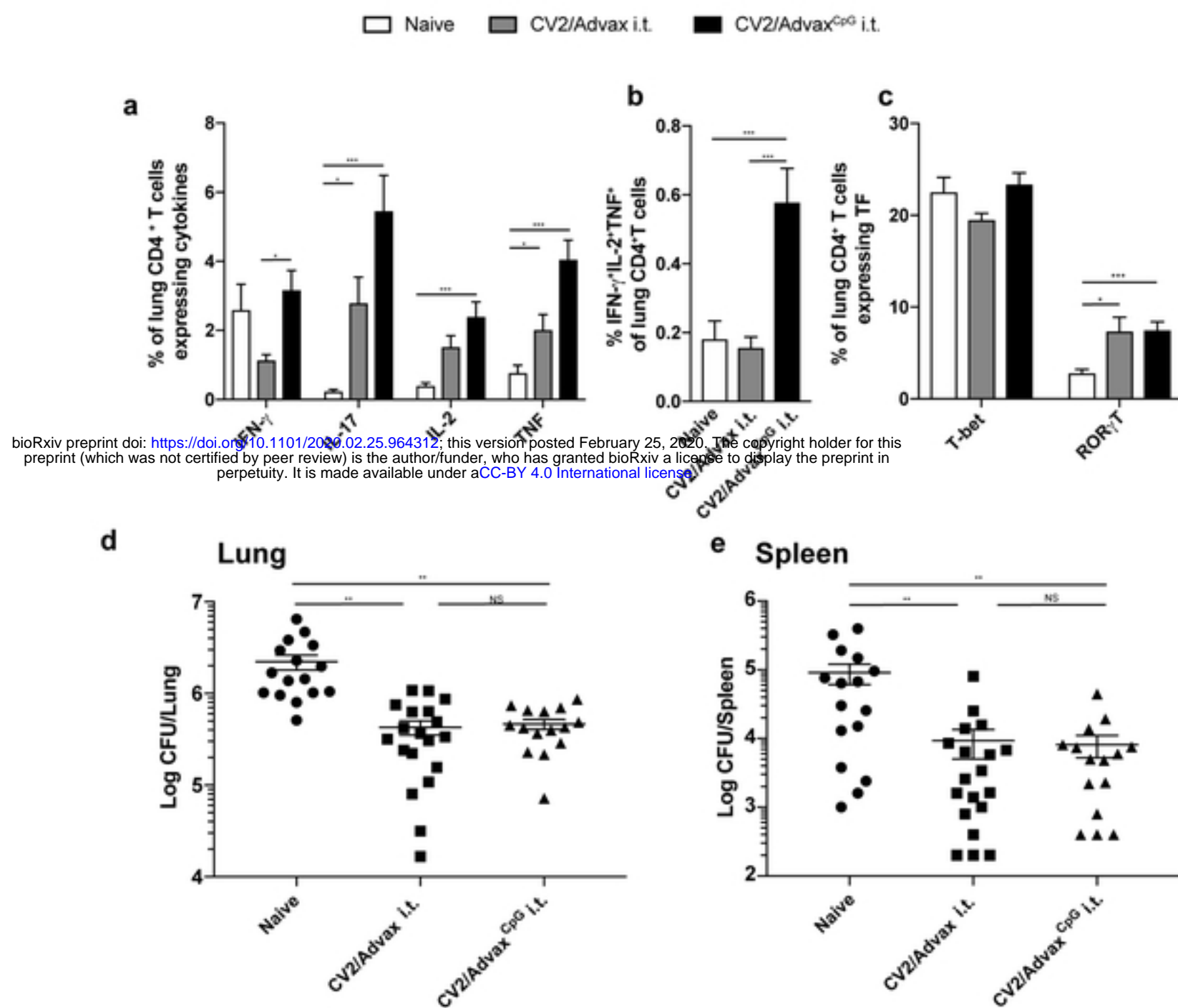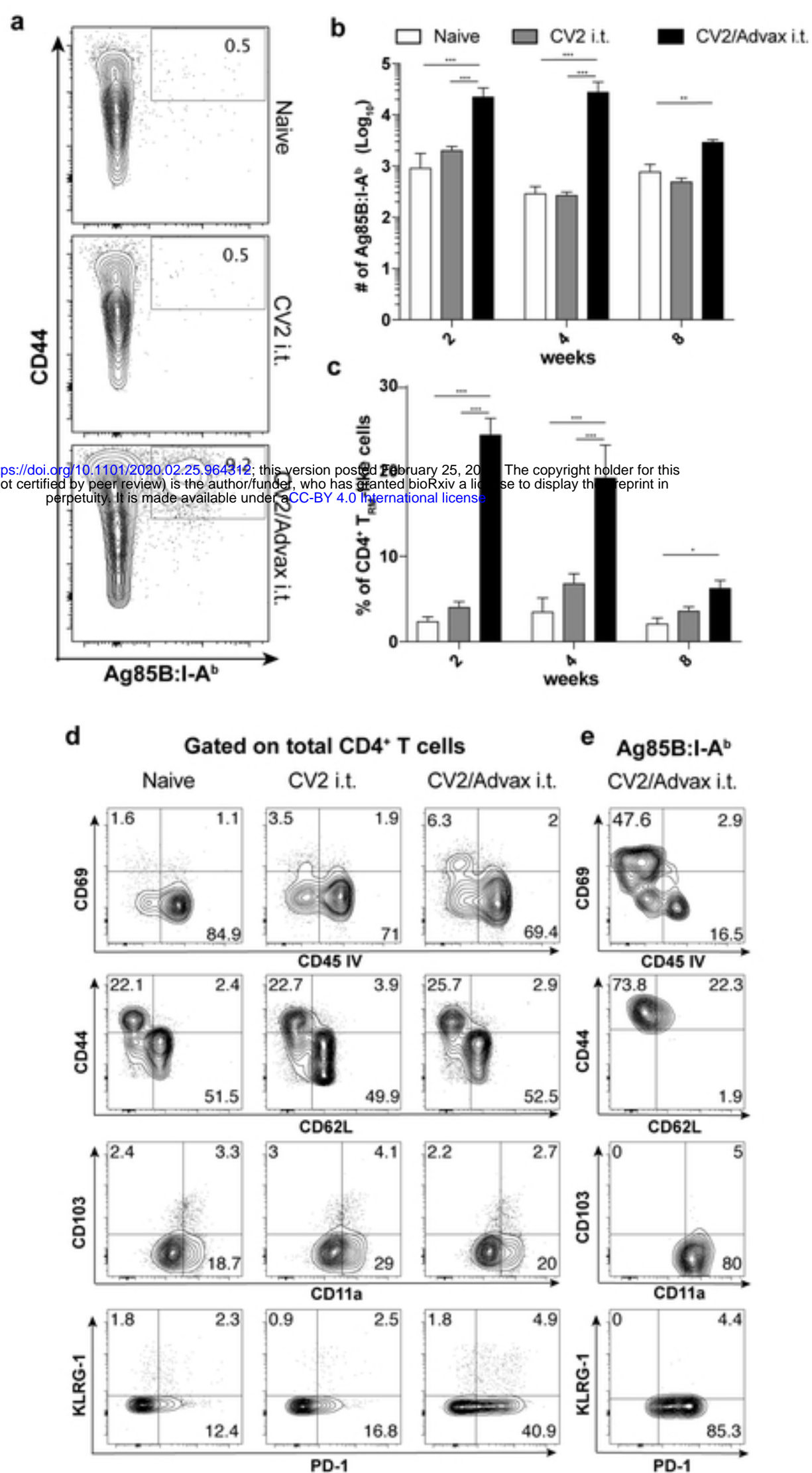725 **S1 Table. List of antibodies used.**

Figure 1, Counoupas *et al*

Figure 2, Counoupas *et al*
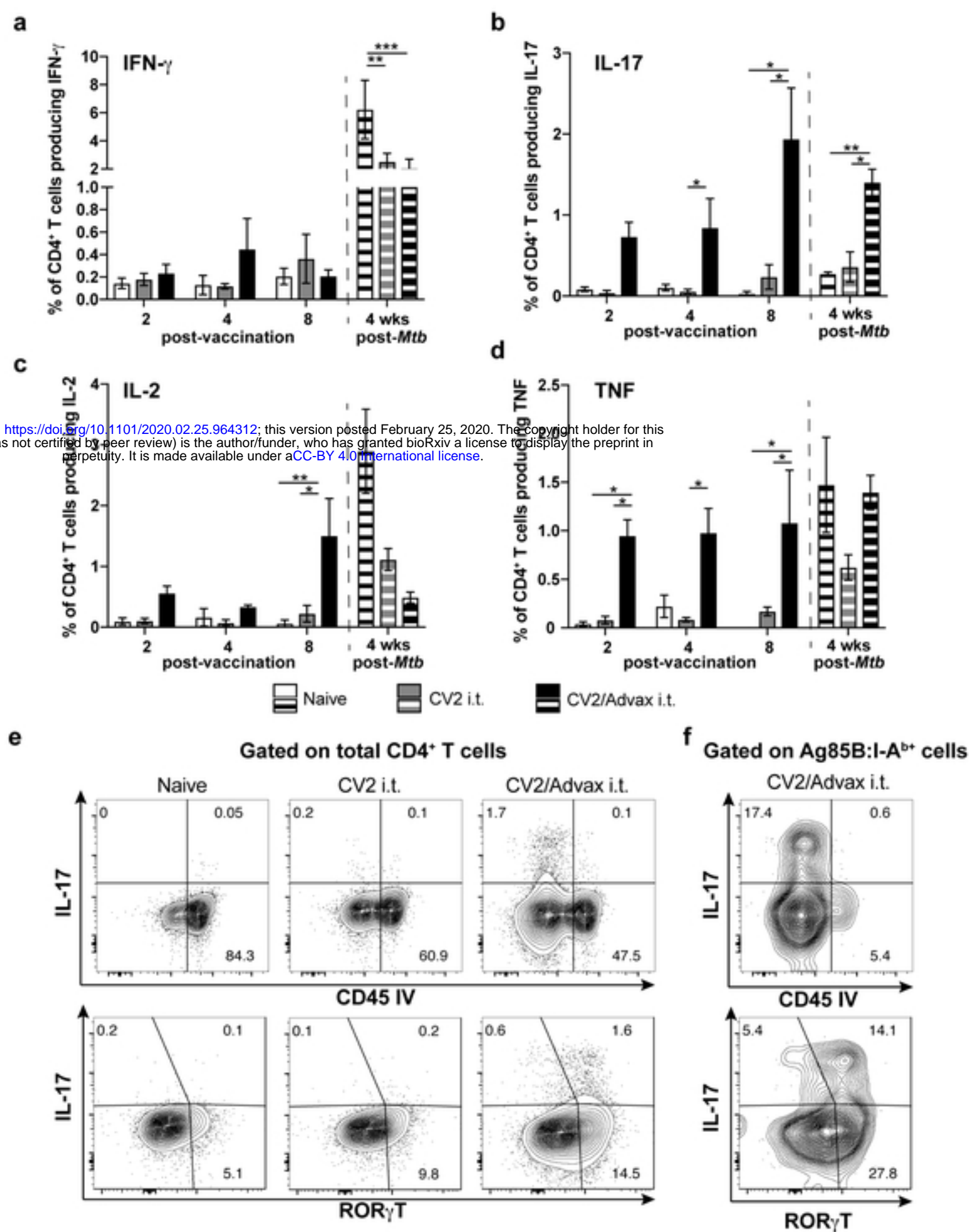
Figure 3, Counoupas *et al*
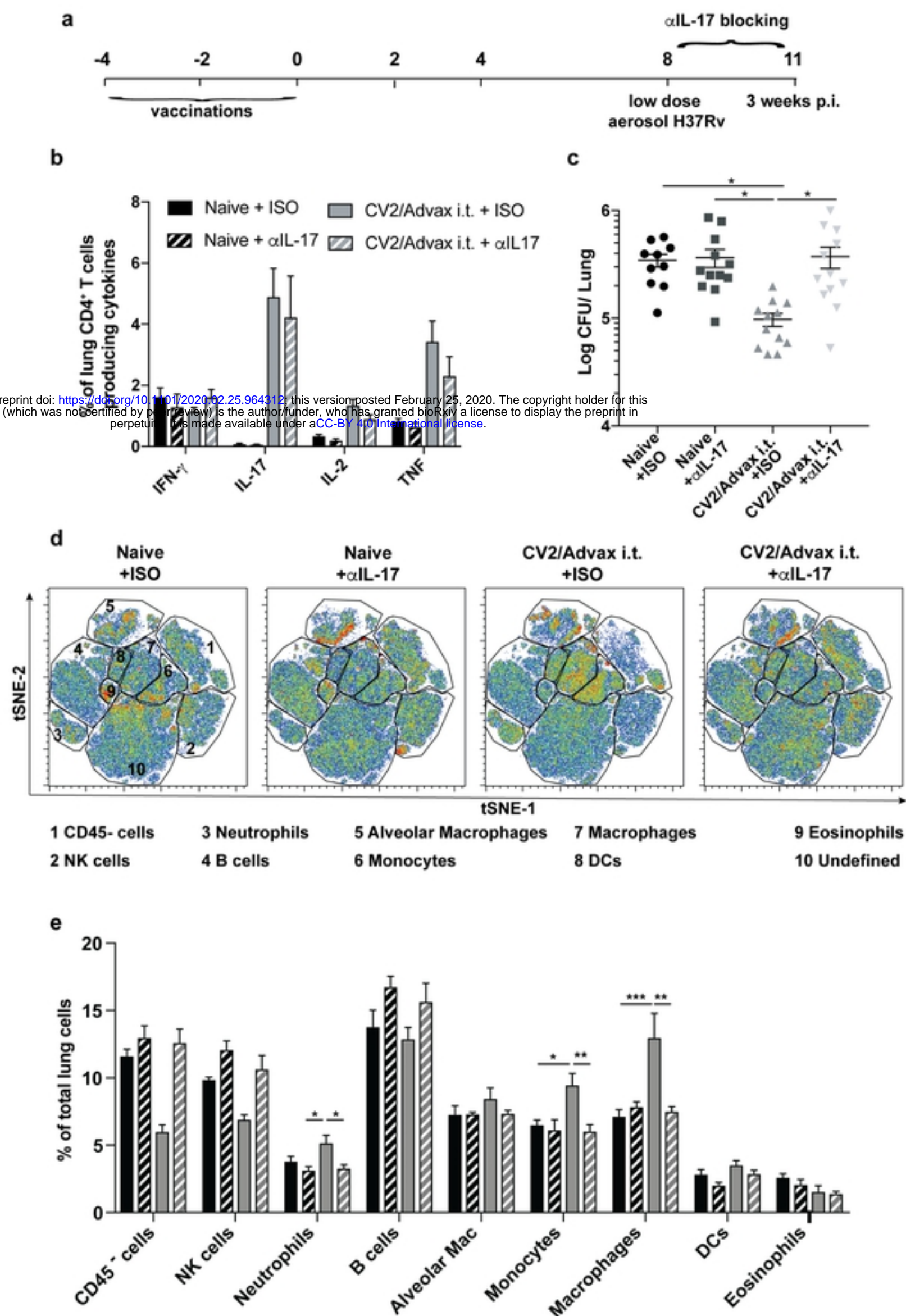
Figure 4, Counoupas *et al*

a

αIL-17 blocking

-4   -2   0   2   4   8   11

vaccinations

low dose   3 weeks p.i.
aerosol H37Rv

b

Legend:
- Naive + ISO
- Naive + αIL-17
- CV2/Advax i.t. + ISO
- CV2/Advax i.t. + αIL17

Y-axis: of lung CD4+ T cells roducing cytokines

X-axis: IFN-γ, IL-17, IL-2, TNF

c

Y-axis: Log CFU/ Lung

X-axis: Naive +ISO, Naive +αIL-17, CV2/Advax i.t. +ISO, CV2/Advax i.t. +αIL-17

d

| Naive +ISO | Naive +αIL-17 | CV2/Advax i.t. +ISO | CV2/Advax i.t. +αIL-17 |

tSNE-2 / tSNE-1

1 CD45- cells   3 Neutrophils   5 Alveolar Macrophages   7 Macrophages   9 Eosinophils

2 NK cells   4 B cells   6 Monocytes   8 DCs   10 Undefined

e

Y-axis: % of total lung cells

X-axis: CD45⁻ cells, NK cells, Neutrophils, B cells, Alveolar Mac, Monocytes, Macrophages, DCs, Eosinophils

Figure 5, Counoupas *et al*

a

Naive +ISO Naive +αIL17 CV2/Advax i.t. +ISO CV2/Advax i.t. +αIL17

Rorγ T / Ki-67

7.2   8.4   10.4   8.1

b

Ki67+ CD4+ T cells (x 10⁻³)

***

Naive +ISO | Naive +αIL-17 | CV2/Advax i.t. +ISO | CV2/Advax i.t. +αIL-17

c

Ki67+Rorγ T+ CD4+ T cells (x 10⁻³)

** **

Naive +ISO | Naive +αIL-17 | CV2/Advax i.t. +ISO | CV2/Advax i.t. +αIL-17

d

Naive +ISO Naive +αIL17 CV2/Advax i.t. +ISO CV2/Advax i.t. +αIL17

CD44 / Ag85B:I-Aᵇ

0.1   0.1   0.1   0.1

CD44 / ESAT6:I-Aᵇ

0.04   0.04   0.20   0.03

e

Ag85B:I-Aᵇ cells (x10⁻³)

*** NS

Naive +ISO | Naive +αIL-17 | CV2/Advax i.t. +ISO | CV2/Advax i.t. +αIL-17

f

ESAT6:I-Aᵇ cells (x10⁻³)

* *

Naive +ISO | Naive +αIL-17 | CV2/Advax i.t. +ISO | CV2/Advax i.t. +αIL-17

Figure 6, Counoupas *et al*

Figure 7, Counoupas *et al*