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

59 **Introduction**

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

71

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

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].
89
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.
105
106

107 **Results**

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

110 Previous studies of intramuscular (i.m.) vaccination of mice with CysVac2/Advax^{CpG} demonstrated
111 substantially enhanced systemic CD4^{+} T cell responses composed of multifunctional Th1 polarized
112 cells, which correlated with protection against aerosol *M. tuberculosis* infection[16]. In this study, we
113 evaluated if delivery to the lung by intratracheal (i.t.) instillation of this vaccine candidate could
114 improve the level of protection induced by this vaccine. Mice were vaccinated by either the i.t. or i.m.
115 routes with CysVac2/Advax^{CpG} 3 times, 2 weeks apart (Fig 1a). When the vaccine-specific T cell
116 response was examined in the blood prior to *M. tuberculosis* challenge, a higher level of circulating
117 polyfunctional CD4^{+} T cells expressing IFN-γ were present after i.m. vaccination (Fig 1b, S1a Fig),
118 with the most prominent phenotype identified as multi-cytokine secreting CD44^{+} CD4^{+} T cells (Fig
119 1c). After i.t vaccination with CysVac2/Advax^{CpG}, PBMC-derived CD4^{+} T cell expressing either IL-
120 2, TNF or IL-17 were more prominent when compared to the i.m route (Fig 1b). Both vaccination
121 regimens induced similar proportions of T-bet expression in circulating CD4^{+} T cells, however i.t.
122 vaccination induced a higher proportion of cells expressing RORγT (Fig 1d).

123

124 The pattern of CD4^{+} T cells immune responses pre-*M. tuberculosis* challenge was compared to that
125 after *M. tuberculosis* infection. In i.m. vaccinated mice, the greatest frequency of CD4^{+} T cells
126 observed were those secreting IFN-γ or TNF (Fig 1e) and this was dominated by cells with a
127 polyfunctional Th1 responses (IFN-γ^{+}IL-2^{+}TNF^{+}, Fig 1f). I.t. vaccination with CysVac2/Advax^{CpG}
128 resulted in a high frequency of CD4^{+} T cells secreting either IL-17 or TNF (Fig 1e) or both cytokines
129 (S1b Fig); these T cell subsets were not observed after i.m. vaccination (Fig 1e, S1 Fig). Further, the
130 frequency of CD4^{+} T cells expressing RORγT was significantly enhanced after i.t. vaccination
131 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.
134
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

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.
180
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 $ROR\gamma T^+$ CD45 $IV^-IL\text{-}17^+CD4^+$ 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

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

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.

321 **Materials and Methods**

322 **Ethics statement**

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

330

331 **Bacterial strains**

332 *M. tuberculosis* H37Rv (BEI Resources, USA) and BCG Pasteur were grown at 37° C in Middlebrook
333 7H9 medium (Becton Dickinson, BD) supplemented with 0.5 % glycerol, 0.02 % Tyloxapol, and 10 %
334 albumin-dextrose-catalase (ADC) or on solid Middlebrook 7H11 medium (BD) supplemented with
335 oleic acid–ADC.

336

337 **Mouse immunization, treatments and infection**

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

347

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^{TM} 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^{TM} 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

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395

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

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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

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 $ROR\gamma T^+$ (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

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,

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.**

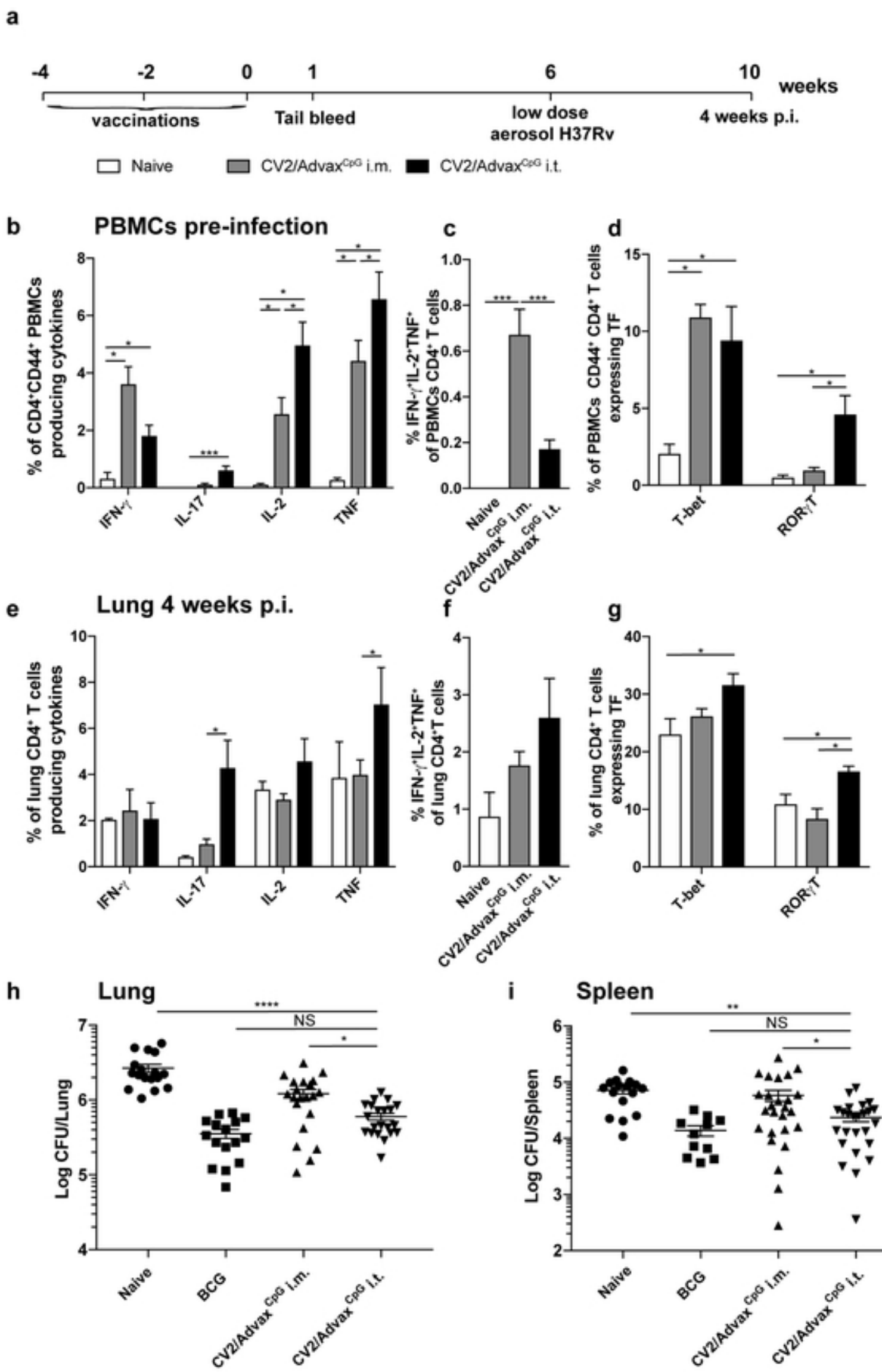

Figure 1, Counoupas *et al*

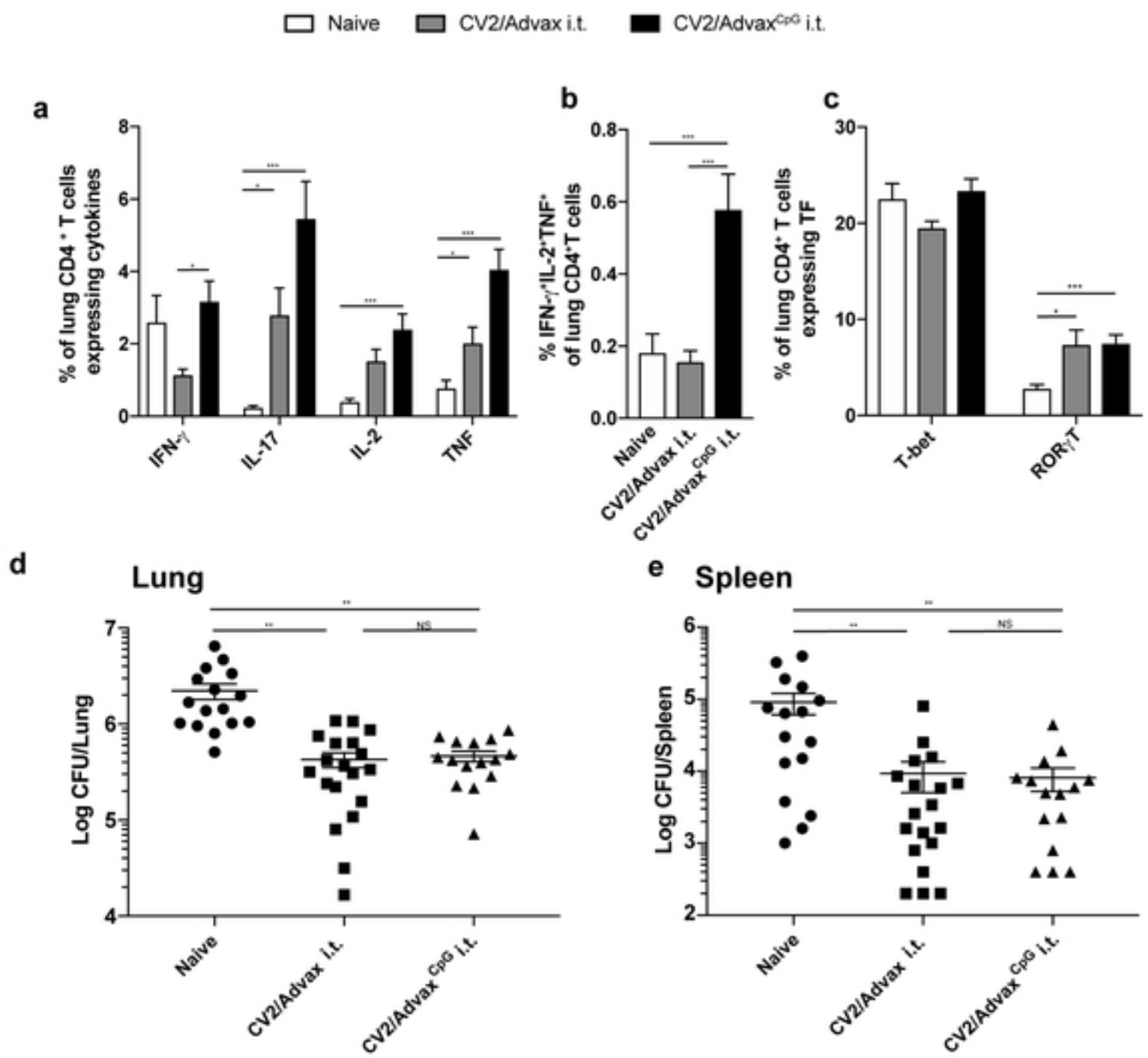

Figure 2, Counoupas *et al*

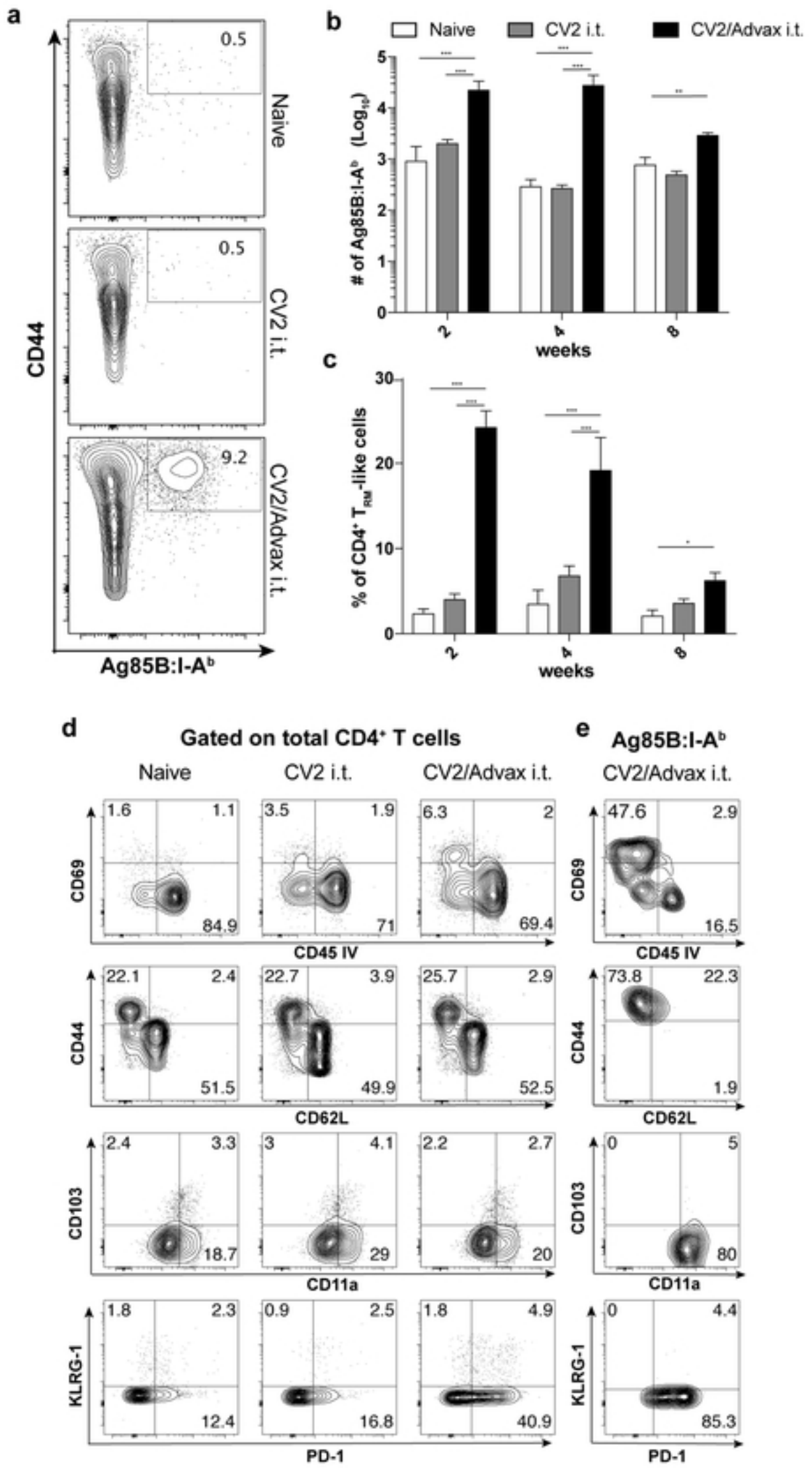

Figure 3, Counoupas *et al*

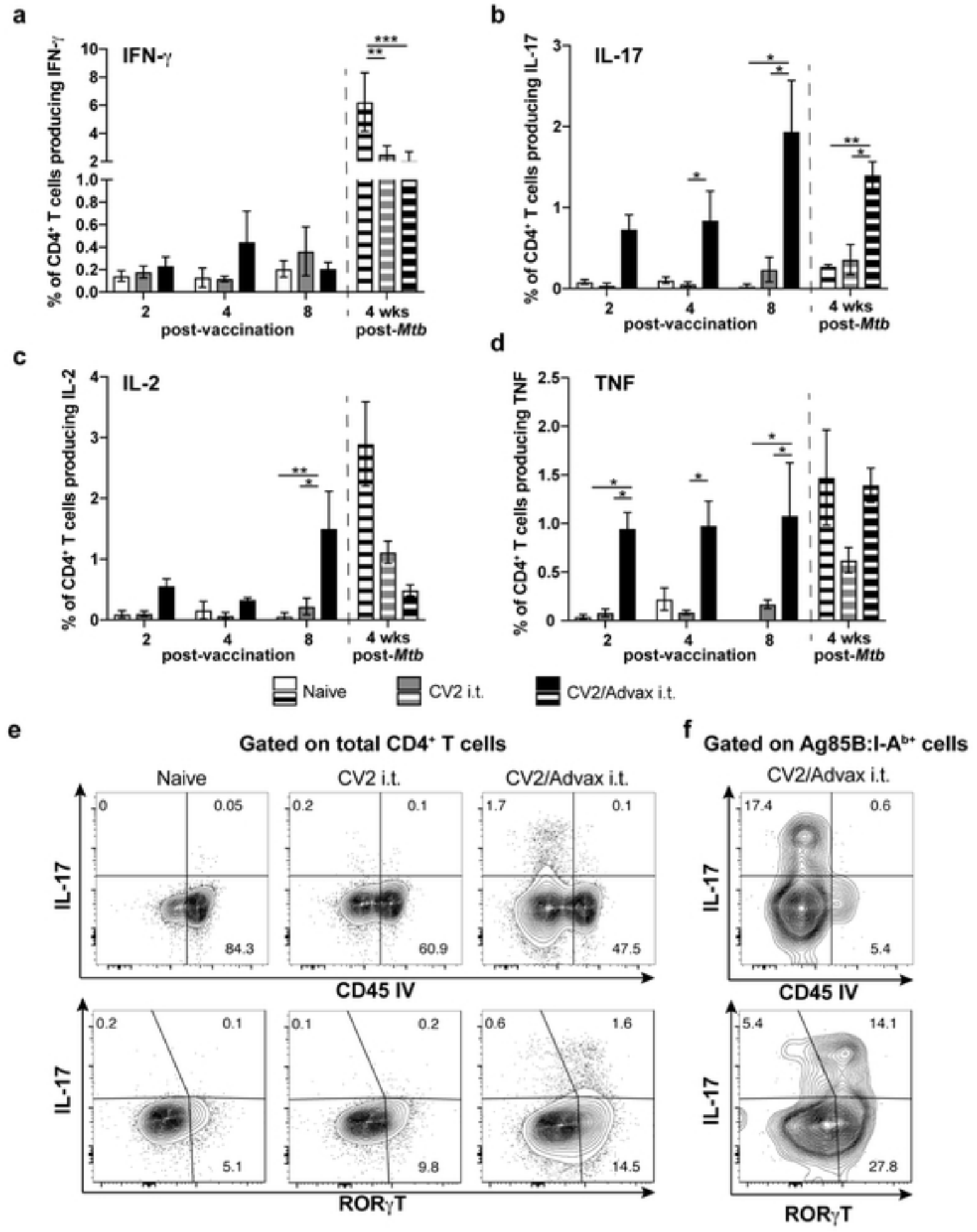

Figure 4, Counoupas *et al*

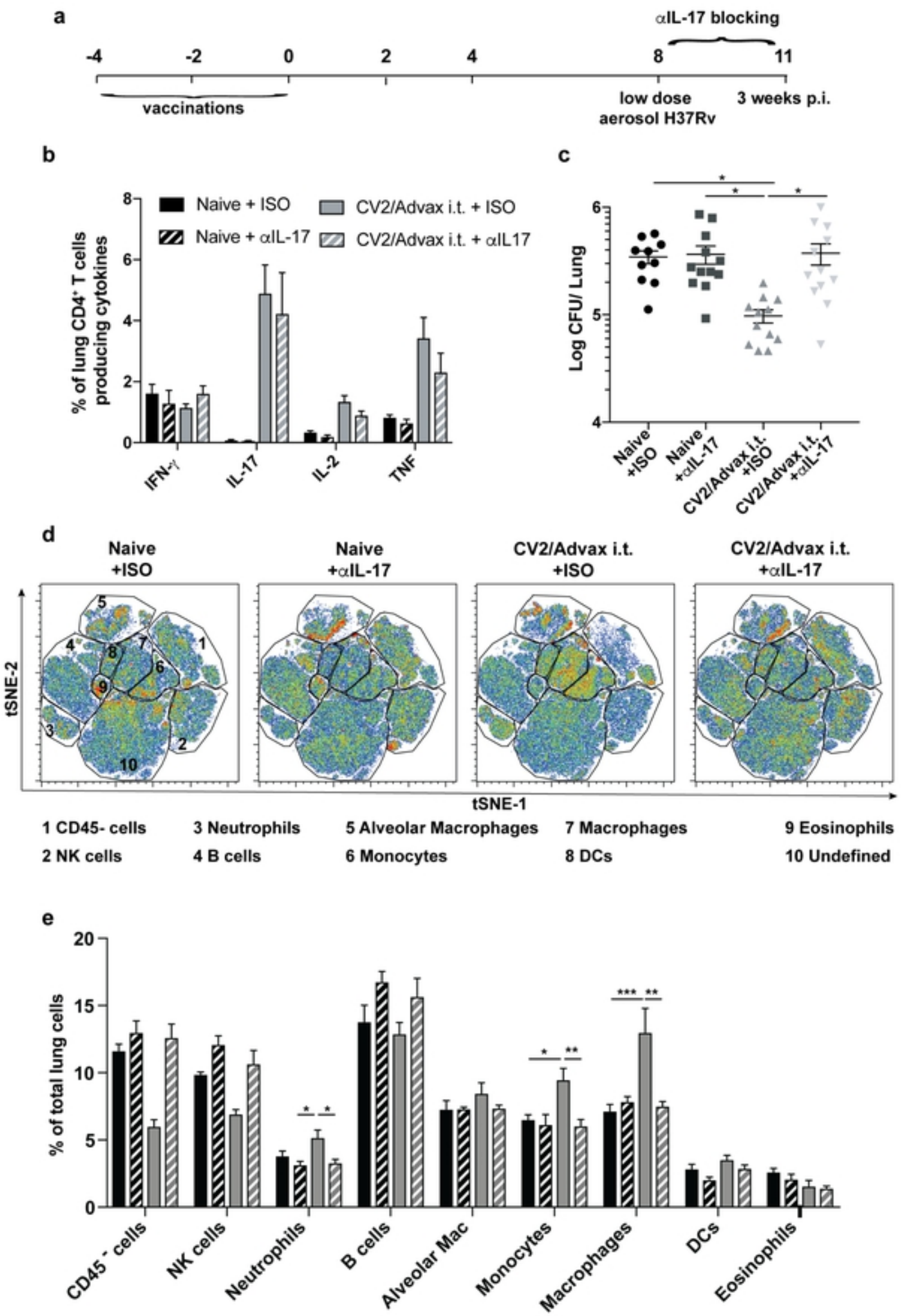

Figure 5, Counoupas *et al*

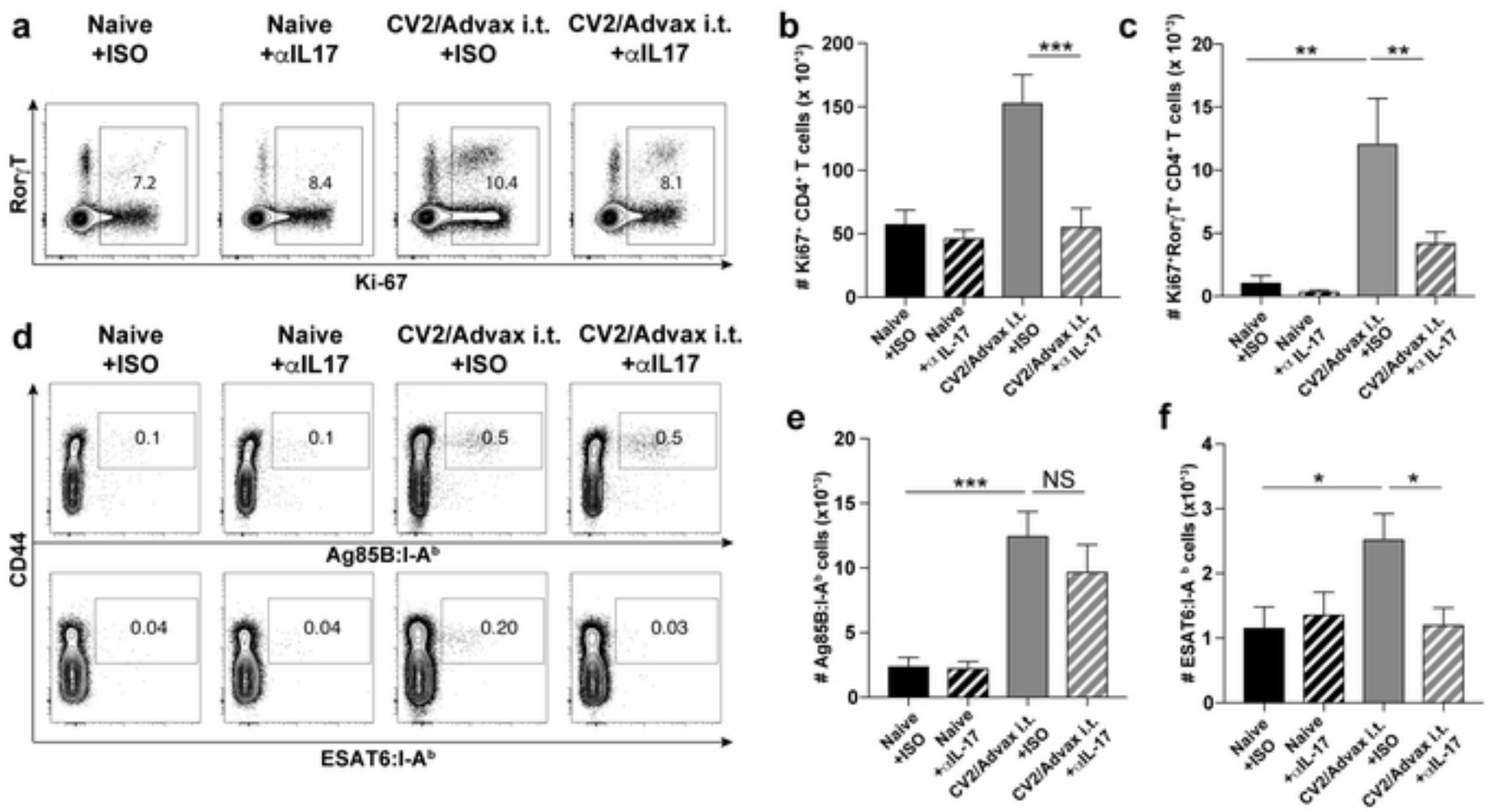

Figure 6, Counoupas *et al*

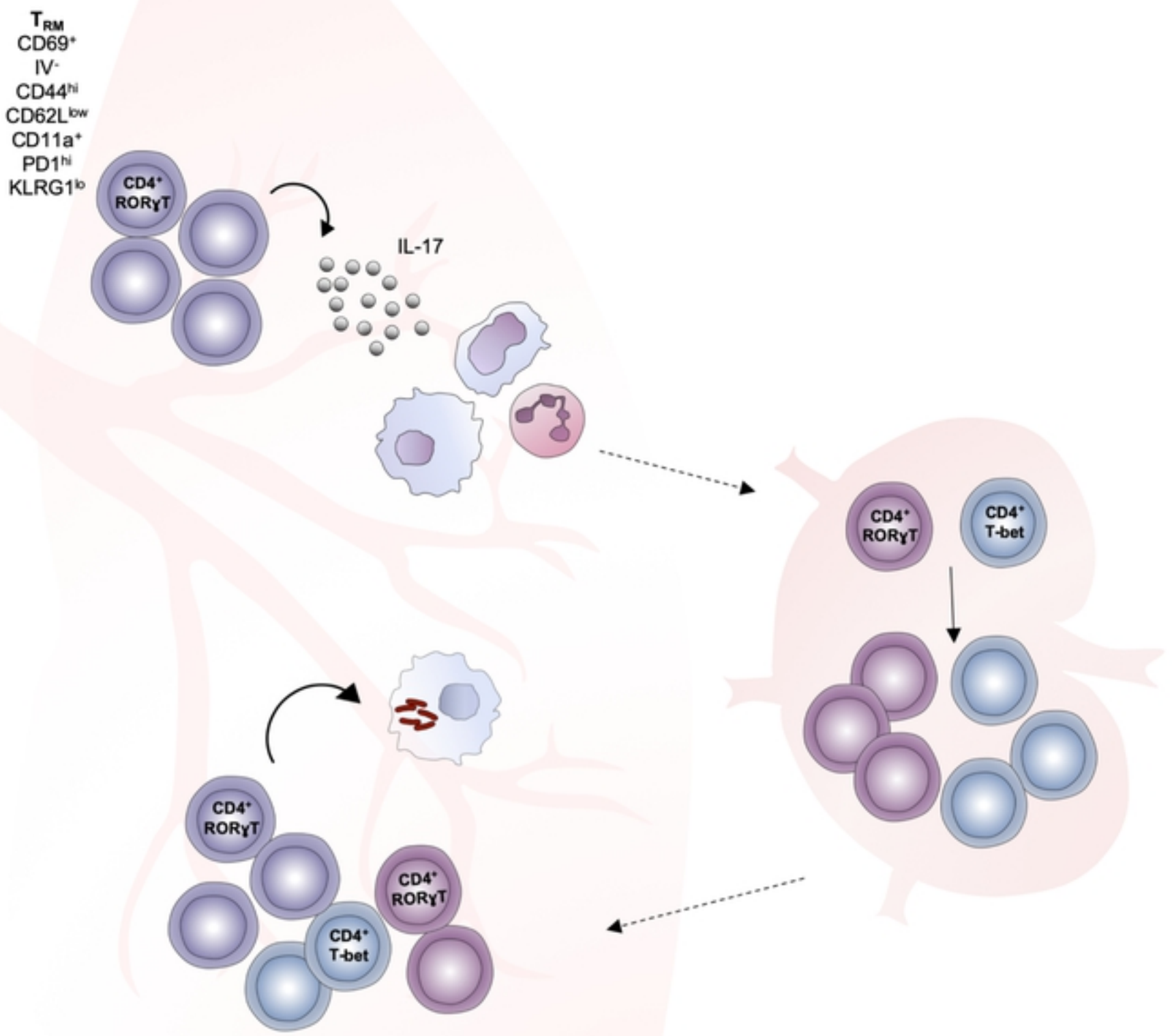

Figure 7, Counoupas *et al*