

1 Control of Mycobacterium tuberculosis Infection in Lungs is Associated with Recruitment of
2 Antigen-Specific Th1 and Th17 cells Co-expressing CXCR3 and CCR6.

3

4 Uma Shanmugasundaram¹, Allison N Bucsan^{2,%}, Shashank R. Ganatra^{2,5,6}, Chris Ibegbu^{1,4},
5 Melanie Quezada¹, Robert V Blair³, Xavier Alvarez^{2,3}, Vijayakumar Velu^{1,4}, Deepak Kaushal
6 ^{2,5,6*} and Jyothi Rengarajan^{1,4,7*}

7

8 1. Emory Vaccine Center, Emory University, Atlanta, GA 30322, USA.

9 2. Tulane National Primate Research Center, Tulane University School of Medicine,
10 Covington, LA, 70433, USA.

11 3. Division of Comparative Pathology, Tulane National Primate Research Center,
12 Covington, LA, 70433, USA.

13 4. Yerkes National Primate Research Center, Emory University, Atlanta GA

14 5. Southwest National Primate Research Center, San Antonio, TX, 78227, USA.

15 6. Texas Biomedical Research Institute, San Antonio, TX, 78227, USA.

16 7. Division of Infectious Disease, Department of Medicine, Emory University School of
17 Medicine

18

19 % current address: Henry M. Jackson Foundation for the Advancement of Military Medicine,
20 Bethesda MD 20817.

21 *Correspondence: Jyothi Rengarajan, PhD, Email: jrengar@emory.edu and Deepak Kaushal,
22 PhD, Email: dkaushal@txbiomed.org

23 **Conflict of interest statement:** The authors have declared that no conflict of interest exists.

24

25

26

27 **Abstract**

28 *Mycobacterium tuberculosis* (Mtb)-specific T cell responses associated with immune control
29 during asymptomatic latent tuberculosis infection (LTBI) remain poorly understood. Using a non-
30 human primate (NHP) aerosol model, we studied the kinetics, phenotypes and functions of Mtb
31 antigen-specific T cells in peripheral and lung compartments of Mtb-infected asymptomatic
32 rhesus macaques by longitudinally sampling blood and bronchoalveolar lavage (BAL), for up to
33 24 weeks post-infection. We found significantly higher frequencies of Mtb-specific effector and
34 memory CD4 and CD8 T cells producing IFN- γ in the airways compared to peripheral blood,
35 which were maintained throughout the study period. Moreover, Mtb-specific IL-17+ and IL-
36 17/IFN- γ double-positive T cells were present in the airways but were largely absent in the
37 periphery, suggesting that balanced mucosal Th₁/Th₁₇ responses are associated with LTBI. The
38 majority of Mtb-specific CD4 T cells that homed to the airways expressed the chemokine
39 receptor CXCR3 and co-expressed CCR6. Notably, CXCR3+CD4+ cells were found in
40 granulomatous and non-granulomatous regions of the lung and inversely correlated with Mtb
41 burden. Our findings provide novel insights into antigen-specific T cell responses associated
42 with asymptomatic Mtb infection that are relevant for developing better strategies to control TB.

43

44 **Key words:** *Mycobacterium tuberculosis*, tuberculosis, Mtb antigen-specific T cells, Latent TB
45 infection, Nonhuman Primate, Rhesus macaque, Bronchoalveolar Lavage, airways, CXCR3,
46 CCR6, granuloma, blood, lung.

47

48 **Introduction**

49 After close contact with a person with active tuberculosis (TB), only a minority of individuals
50 develops primary TB disease. The majority of individuals successfully control *Mycobacterium*
51 *tuberculosis* (Mtb) infection in a clinically asymptomatic state termed latent TB infection
52 (LTBI)(1). Individuals with LTBI are defined as having a positive tuberculin skin test (TST)
53 and/or Interferon-gamma Release Assay (IGRA), a normal chest radiograph and the absence of
54 clinical signs and symptoms of disease(2). Latently-infected individuals are generally thought to
55 contain Mtb within granulomatous lesions in the lung without completely eradicating bacteria,
56 although direct evidence for the persistence of Mtb in human LTBI is lacking. Moreover, it is
57 increasingly recognized that clinically asymptomatic individuals likely reflect a spectrum of
58 infection outcomes, ranging from individuals who may have eliminated infection, to those with
59 low levels of replicating or non-replicating persistent bacteria, to individuals who may harbor
60 actively replicating bacteria without exhibiting overt clinical symptoms(3-5). Identifying immune
61 responses associated with asymptomatic Mtb infection states will provide key insights into
62 mechanisms of immune control that protect against progressing to active TB disease.

63

64 Antigen-specific T cell responses are critical for immune control of Mtb infection. In response to
65 Mtb infection, the majority of infected people mount robust CD4 T cell responses involving T
66 helper 1 (Th₁) cytokines such as IFN- γ and TNF- α , which are important for activating
67 macrophages and curtailing Mtb replication in the lung(6, 7). In addition, IL-17 and Th₁₇
68 responses have emerged as important for protective immunity against TB, but mucosal Mtb-
69 specific Th₁₇ responses during immune control of LTBI in humans remain poorly studied. In
70 general, the nature and kinetics of Mtb antigen-specific T cell responses in the blood and lung
71 compartments during human LTBI have not been well characterized. This is in part because
72 small animal models do not reproduce key aspects of human LTBI. Moreover, accurately
73 documenting Mtb exposure, initial infection and early events following infection in humans is

74 almost impossible. Thus, studies of Mtb antigen-specific T cells in humans have been largely
75 confined to cross-sectional characterization of peripheral responses in the blood(8-12). While
76 some studies have examined responses in bronchoalveolar lavage (BAL)(13-15), longitudinal
77 studies in humans comparing Mtb antigen-specific T cell responses in blood and lung
78 compartments have been lacking.

79
80 Nonhuman primate (NHP) macaque models of Mtb infection recapitulate multiple features of
81 human Mtb infection, including clinically asymptomatic infection and symptomatic active TB
82 disease (16-18) and are attractive for studying immune parameters associated with control of
83 Mtb infection in peripheral blood and lung compartments. We have previously established a
84 model of LTBI in Indian rhesus macaques, in which low-dose aerosol infection with Mtb
85 CDC1551 leads to the development of asymptomatic Mtb infection. Approximately 80% of
86 infected animals remained disease-free for up to 6 months post-infection (19-24) while only
87 ~20% progressed to active TB disease. In this study, we characterized the nature, magnitude
88 and kinetics of Mtb antigen-specific CD4 and CD8 T cell responses during asymptomatic LTBI
89 in rhesus macaques over the course of ~24 weeks post-infection, by serially sampling blood and
90 lung compartments in conjunction with intensive clinical monitoring. We found significantly
91 higher frequencies of Mtb-specific effector and memory CD4 and CD8 T cells producing IFN- γ in
92 the airways compared to peripheral blood; and these were maintained throughout the 24-week
93 study period. Moreover, Mtb-specific IL-17+ and IL-17/IFN- γ double-positive T cells were
94 present in the airways but were largely absent in peripheral blood. The majority of Mtb-specific
95 CD4 T cells that homed to the airways expressed the chemokine receptor CXCR3 and co-
96 expressed CCR6. Notably, CXCR3+CD4+ cells were also found in the lungs of animal with LTBI
97 and active TB and were associated with lower Mtb burdens. Our findings provide new insights
98 into antigen-specific T cell responses associated with the establishment and maintenance of
99 asymptomatic infection.

100 **Results**

101 **Experimental design and clinical characteristics of rhesus macaques with asymptomatic** 102 **Mtb infection.**

103 Six animals with no clinical signs or symptoms of disease were studied over the course of ~24
104 weeks following low-dose aerosol infection (**Figure 1A**). These animals had a median chest
105 radiograph (CXR) score of 0.4, denoting no pulmonary lesions, and maintained normal CRP
106 levels (**Figure 1B**), body weight (**Supplementary Figure 1A**) and temperature (**Supplementary**
107 **Figure 1B**). All animals except one had detectable bacteria upon plating BAL (**Figure 1C**) and
108 three of these animals had detectable, albeit low (< 4-logs), lung bacterial loads at necropsy
109 (**Figure 1D**). Examination of Hematoxylin-Eosin (H&E)-stained lung tissue at necropsy (at ~24
110 weeks post-infection) showed that animals harbored varying degrees of inflammatory lesions,
111 ranging from 0.2% to 20% of the lung being constituted by granulomas (**Figure 1E**). These
112 results highlight the heterogeneity of clinically asymptomatic animals that control Mtb infection,
113 consistent with the idea that asymptomatic LTBI is represented by a spectrum of Mtb infection
114 states(3, 5).

115 **Comparison of total CD4 and CD8 T cell frequencies in PBMC and BAL of asymptomatic** 116 **rhesus macaques.**

117 We assessed the frequencies of total CD4 and CD8 T cells before Mtb infection (week -1) and
118 at 3, 7, 11, 15, 19 and 24 weeks post-infection in the six animals with asymptomatic Mtb
119 infection (**Figure 2**). We observed significantly higher frequencies of CD4 T cells in peripheral
120 blood compared to BAL at all time points studied ($p=0.01$). In contrast, higher frequencies of
121 CD8 T cells were present in BAL compared to peripheral blood at all time points ($p=0.01$).
122 Overall, CD4 and CD8 T cells were each maintained over time in both BAL and PBMCs during
123 the course of the study.

124 **High frequencies of Mtb antigen-specific CD4 and CD8 T cells producing IFN- γ are**
125 **maintained in the BAL of rhesus macaques during asymptomatic Mtb infection.**

126 To assess the kinetics of Mtb antigen-specific CD4 and CD8 T cell responses in the peripheral
127 blood and airways of asymptomatic animals that control Mtb infection as LTBI, we stimulated
128 PBMC and BAL samples at each time point with Mtb cell wall antigens (CW) and ESAT-6/CFP-
129 10 peptide pools, followed by intracellular cytokine staining (ICS) and flow cytometry to assess
130 production of IFN- γ . In both PBMC and BAL, CW- and ESAT-6/CFP-10-specific, IFN- γ -
131 producing CD4 (**Figure 3A & B, Supplementary Figure 2A**) and CD8 (**Figure 3C & D,**
132 **Supplementary Figure 2B**) T cells were detected as early as 3 weeks post-Mtb infection. Mtb-
133 specific CD4 and CD8 T cell frequencies increased at week 7 in both PBMC and BAL and were
134 maintained up to the necropsy endpoint. Overall, while frequencies of Mtb-specific IFN- γ + CD4
135 were higher than their CD8 counterparts, both CD4 and CD8 T cell frequencies were
136 significantly ($p=0.03$) higher in BAL compared to peripheral blood at all time points [mean \pm SEM,
137 at week 7 post Mtb-infection, 0.3% \pm 0.06 in PBMC and 15% \pm 6.5 in BAL]. Thus, robust Mtb
138 antigen-specific CD4 and CD8 T cell responses are maintained in lung compartments during
139 asymptomatic LTBI.

140 **High proportions of CD28+CD95+ Mtb-specific memory T cells in peripheral blood and**
141 **BAL.**

142 The proliferation of antigen-specific T cells in response to infection leads to development of a
143 pool of antigen-experienced memory T cells that are important for mediating effective protection
144 against re-challenge(25). TB vaccine strategies aim to elicit protective antigen-specific memory
145 T cell responses(26, 27). Moreover, memory T cells are thought to play an important role in
146 controlling Mtb infection during LTBI but remain poorly studied in lung compartments. We
147 therefore investigated Mtb-specific effector and memory CD4 and CD8 T cell subsets in

148 asymptomatic rhesus macaques by assessing their differentiation state, based on cell surface
149 expression of CD28 and CD95 on IFN- γ + CD4 T cells and CD8 T cells (**Figure 4**). At week 7
150 post-infection we observed that antigen-specific IFN- γ + CD4 and CD8 T cells in both PBMC and
151 BAL were predominantly CD28+CD95+ (**Figure 4A-D**), indicating a central memory-like
152 phenotype. Moreover, these memory T cells were maintained at high levels throughout the time
153 course of the study. While we observed higher proportions of antigen-specific CD28-CD95+
154 effector CD8 T cells compared to CD4 T cells in the BAL (**Figure 4C, D**), the relative proportions
155 of CD28+CD95+ and CD28-CD95+ CD4 and CD8 T cell subsets were maintained throughout
156 latent infection in both peripheral blood and airways (**Figure 4E-H**). Our data suggest that
157 antigen-specific memory T cells in the blood and lung compartments are long-lived and are
158 likely to contribute towards maintaining immune control during LTBI.

159 **IL-17+ and IFN- γ /IL-17+ T cells are present in the BAL but not in PBMCs from**
160 **asymptomatic rhesus macaques.**

161 In addition to IFN- γ production, IL-17 and Th₁₇ responses have emerged as important for
162 protective immunity against TB. To determine whether Mtb-specific IL-17 producing T cells are
163 present during LTBI, we next assessed the kinetics of IL-17+ CD4 and CD8 T cells in both BAL
164 and PBMC (**Figure 5 A-D**). We observed minimal to no Mtb-specific IL-17+ CD4 and CD8 T cell
165 responses in PBMCs throughout the time course of the study (**Figure 5 A-D, Supplementary**
166 **Figure 2C & D**). In contrast, CW- and ESAT-6/CFP10-specific IL-17+ CD4 (**Figure 5A & B**)
167 and CD8 cells (**Figure 5C & D**) T cells were clearly detectable in the BAL. Thus, the presence
168 of Mtb-specific IL-17+ Mtb-specific T cells in the airways suggests preferential accumulation of
169 IL-17-producing T cells at lung mucosal sites of Mtb infection and replication.

170 Although IL-17 is a hallmark of Th₁₇ cells, IL-17/IFN- γ double-positive T cells are also known to
171 be present at mucosal sites of inflammation(28). We sought to investigate whether Mtb-specific

172 CD4 T cells that express both IFN- γ and IL-17 are present in BAL (**Figure 6A**). At week 7 post-
173 Mtb infection, in addition to CW- and ESAT-6/CFP-10- stimulated CD4 T cells that singly-
174 expressed either IFN- γ and IL-17 (**Figure 6A-C**), we found Mtb-specific T cells that co-
175 expressed IFN- γ and IL-17 (**Figure 6D**). While these IFN- γ /IL-17 double positive cells were
176 present at relatively low frequencies, our results are consistent with recently published data
177 showing that rhesus macaques who were protected from developing TB following mucosal
178 vaccination with BCG, harbored mycobacteria-specific IFN- γ /IL-17 double-positive CD4 T
179 cells(28). Together, the data represented in **Figures 5 and 6** show that in addition to Mtb
180 antigen-specific IFN- γ + T cells, Mtb-specific IL-17+ and IFN- γ /IL-17 double-positive T cells are
181 present in the airways of asymptomatic rhesus macaques, suggesting an association between
182 balanced Th₁/Th₁₇ responses at mucosal sites of infection and immune control of Mtb infection.

183 **Mtb-specific IFN- γ + and IL-17+ CD4 T cells in the airways co-express CXCR3 and CCR6.**

184 Chemokine receptors CXCR3 and CCR6 regulate the migration of antigen-specific Th₁ and Th₁₇
185 cells, respectively, into inflamed mucosal tissues following microbial infection(29-31). We
186 therefore sought to determine whether these chemokine receptors were expressed on the Mtb-
187 specific IFN- γ + and IL-17+ CD4 T cells present in the airways of macaques that controlled Mtb
188 infection. We assessed the frequencies of CW- and ESAT-6/CFP-10-specific IFN- γ + and IL-17+
189 CD4 T cells in the BAL that expressed either CXCR3 or CCR6 (**Figure 7A, B**) and found that
190 the majority of IFN- γ - and IL-17-producing CD4 T cells expressed CXCR3 (mean \pm SEM at week
191 7 post-Mtb infection, IFN- γ + 82.4% \pm 2.9, IL-17+ 61% \pm 7) and CCR6 (mean \pm SEM at week 7 post
192 Mtb infection, IFN- γ + 56% \pm 8.6, IL-17+ 67% \pm 11). To assess co-expression of CXCR3 and
193 CCR6, we analyzed samples from the week 7-time point, since the low frequencies of IL-17-
194 producing T cells at later time points precluded reliable analyses. We found that the majority of
195 Mtb-specific IFN- γ + (**Figure 7C, D**) and IL-17+ (**Figure 7E, F**) CD4 T cells in BAL co-expressed
196 CXCR3 and CCR6. Moreover, IFN- γ /IL-17 double-positive cells were also predominantly

197 CXCR3+CCR6+ (**Figure 7G, H**). Overall, CD4 T cells co-expressing CXCR3 and CCR6 were
198 the main IFN- γ - and IL-17-producing subsets present in the airways of macaques with LTBI.

199 **CXCR3+CD4 T cells in the lung correlate with lung Mtb burden.**

200 Our observation that CXCR3 and CCR6 co-expressing cells were the predominant Mtb antigen-
201 specific CD4+ T cells in the airways of macaques with LTBI prompted us to investigate the
202 localization and frequencies of these cells in the lungs of Mtb-infected NHP by immunostaining
203 of lung tissue sections. While CXCR3+ CD4 T cells were clearly detected in lung tissue, robust
204 immunostaining for CCR6 in paraffin-fixed lung tissue could not be established, despite
205 extensive efforts. Since most of the CCR6+ CD4 T cells in BAL were also positive for CXCR3
206 (**Figure 7**), we used CXCR3 as a proxy for both chemokine receptors (**Figure 8**). We also
207 stained lung sections with antibodies to CD163 and CD68 to identify macrophages(32).
208 Immunostaining of lung sections from animals with asymptomatic LTBI showed that CXCR3+
209 CD4 T cells were present in lung tissue (**Figure 8A**), with higher densities of CXCR3+ CD4 T
210 cells in granulomatous areas of the lung compared to non-granulomatous areas (**Figure 8B**).
211 Next, to investigate the relationship between lung CD4+CXCR3+ cells and Mtb burden, we
212 stained archived lung tissue sections from rhesus macaques with active TB disease(24).
213 Quantification of the density of CXCR3+CD4+ cells in the lungs of animals with active TB
214 showed that the density of CXCR3+CD4+ cells was significantly ($p=0.03$) higher in the
215 granulomatous areas compared to non-granulomatous areas of the lung, with overall lower
216 CXCR3+CD4+ densities in the lungs of active TB relative to LTBI (**Figure 8B**). Interestingly, we
217 observed a negative correlation between lung CXCR3+CD4+ densities and lung CFU (**Figure**
218 **8C**), suggesting that CXCR3+ CD4 T cells are associated with lower Mtb burdens. These
219 results, along with the results reported in **Figures 6 and 7**, suggest that recruitment of CXCR3-
220 and CCR6-expressing Th₁ and Th₁₇ subsets to the airways and lungs of macaques contributes
221 to immune control of Mtb burden.

222 Discussion

223 Asymptomatic IGRA+ individuals with LTBI can remain disease-free for decades but current
224 tests cannot determine whether they harbor bacteria or have cleared infection. Thus, defining
225 immune responses associated with establishing and maintaining human LTBI remains
226 challenging. NHPs, e.g., macaques, recapitulate multiple aspects of human Mtb infection and
227 disease progression and are attractive animal models for studying LTBI (16, 33-38). We recently
228 showed that rhesus macaques with asymptomatic Mtb infection harbor viable Mtb bacteria in
229 their lungs for up to nine months(20). Moreover, co-infection with simian immunodeficiency virus
230 (SIV) induced reactivation to TB disease while treatment with isoniazid and rifapentine for 3
231 months prevented SIV-mediated reactivation to TB. In the current study, we used our
232 established low-dose aerosol infection model of LTBI to study the nature and kinetics of Mtb
233 antigen-specific T cell responses in six rhesus macaques that remained free of clinical signs
234 and symptoms of TB disease for upto 24 weeks post-infection. We observed varying degrees of
235 lung inflammation and granulomas across all the asymptomatic animals, (**Figure 1E and Figure**
236 **8**), consistent with the heterogeneity reported in cynomolgus macaques with LTBI, where both
237 sterile and non-sterile granulomas were present within a single host (39).

238

239 Previous studies in NHPs assessed antigen-specific immune responses in BAL at early time
240 points macaques in infected macaques (35, 40-42), or during vaccination (43-45) (28).
241 However, in-depth analysis of the phenotypes, functionality and kinetics of Mtb antigen-specific
242 immune responses in blood and BAL during establishment and maintenance of asymptomatic
243 latent infection have been lacking. To our knowledge, our current study, in which we undertook
244 monthly collection of blood and BAL samples starting at week 3 post-infection and up to 24
245 weeks, is the first to longitudinally profile Mtb-specific (CW and ESAT-6/CFP-10-specific) CD4
246 and CD8 T cell responses during LTBI and to directly compare peripheral blood and lung
247 compartments over an extended period of asymptomatic infection. We found that the

248 frequencies of Mtb CW- and ESAT-6/CFP-10-specific IFN- γ - CD4 and CD8 T cell responses
249 were significantly higher in BAL (51-fold for CD4 and 30- fold for CD8) compared to PBMCs,
250 with higher proportions of CD8 T cells present in BAL compared to their CD4 T cell
251 counterparts. The presence of high frequencies of Mtb-specific T cells in the airways as early as
252 3 weeks after Mtb infection, and their persistence throughout the study until 24 weeks, suggests
253 that Mtb-specific T cells accumulate at mucosal sites of infection, which might contribute to
254 control of latent Mtb infection. Lack of IFN- γ + CD4 T cells have been shown to result in loss of
255 Mtb bacterial control in mice (7, 46) and in macaques(47, 48).

256
257 Interestingly, in addition to antigen-specific IFN- γ + CD4 T cells, we also detected Mtb-specific
258 IL-17+ CD4 T cells in the BAL by 3 weeks post-Mtb infection. Mtb-specific IL-17+ cells peaked
259 at week 7 and were detected mainly in BAL, being largely absent in blood. The accumulation of
260 IL-17 cytokine-producing CD4 T cells in lung compartments is consistent with the known
261 preferential accumulation of Th₁₇ cells at mucosal sites during infection(49). Various studies in
262 mice have also suggested that Th₁₇ cells play an important role in protective immunity against
263 TB, both in the context of vaccination (50) as well as in the context of disease progression (51,
264 52). In addition, studies in macaques with active TB disease have suggested an association
265 between granuloma IL-17+ cells and upregulation of genes related to Th₁₇ cells and control of
266 Mtb infection (53, 54). Interestingly, in addition to antigen-specific IL17+ CD4 T cells, we also
267 observed (IFN- γ +IL-17+) double-positive CD4 T cells in the BAL. Our results indicate that both
268 IFN- γ single-positive and IFN- γ +IL-17+ double-positive cells emerge soon after Mtb infection
269 and persist for several weeks, thus likely contributing to controlling Mtb infection during LTBI.
270 These data support and extend previous studies in mice (50, 55, 56) and in macaques (28, 53)
271 suggesting that balanced Th₁ and Th₁₇ responses are associated with enhanced immunity to
272 TB. Future studies that selectively deplete IL-17 or IFN- γ + CD4 T cells in the context of LTBI or
273 vaccination in macaques will provide more direct evidence for the role of these cells in LTBI. In

274 addition, determining the balance between Th₁/Th₁₇ responses in animals that fail to control Mtb
275 infection at early time points post-infection will be of great interest. Overall, our study contributes
276 new insights into the kinetics of Mtb antigen-specific Th₁ and Th₁₇ responses in blood and lung
277 compartments during asymptomatic LTBI in over extended periods of time.

278

279 Elicitation and maintenance of memory responses has been associated with protective
280 immunity(26, 27). In the present study, Mtb antigen specific memory CD4 and CD8 T cells, both
281 in PBMC and BAL, were maintained at high levels throughout the time course of the study,
282 suggesting that these cells are likely to be long-lived and involved in maintenance of the
283 asymptomatic LTBI state. Although CD4 T cells were the predominant subset responding to Mtb
284 infection, we also detected antigen-specific IFN- γ + CD8 T cells, which increased in frequency
285 between 3 and 7 weeks in parallel with antigen-specific CD4 T cells. The CD8 T cell responses
286 that we observed in macaques that control infection differ from previous mouse studies in which
287 lung CD8 T cell responses were considerably delayed compared to CD4 T cell responses (57).
288 Additionally, our study shows that Mtb-specific CD8 T cells producing IL-17 are present,
289 although co-production of IFN- γ and IL-17 was reduced relative to the CD4 subsets. Overall, our
290 findings show that Mtb-specific T cells produce both IFN- γ and IL-17 in the BAL at higher
291 frequencies compared to blood and suggest that these responses may be associated with
292 control of Mtb during asymptomatic Mtb infection.

293

294 Th₁ and Th₁₇ cells can be identified by the expression of chemokine receptors, specifically
295 CXCR3 and CCR6 are considered surface marker for Th1 and Th17 respectively(58). Within the
296 CXCR3 and CCR6 axis, another subset of cells that co-express both CXCR3 and CCR6 have
297 been identified (59) and these cells produce both cytokines IFN- γ and IL-17. These IFN- γ /IL-17
298 double-positive CD4 T cells have been shown to play a pathogenic role during autoimmune
299 diseases(60) but their role in protective immunity to infection remains unclear. With respect to

300 Mtb-infection, a recent study showed preferential expansion of CXCR3+CCR6⁻ and reduction in
301 CXCR3⁻CCR6⁺ CD4 subsets in individuals with TB-IRIS(61). In latently infected-individuals,
302 CD4 T cells that mainly co-express CXCR3 and CCR6 that produced IFN- γ were reported to be
303 present in peripheral blood but these cells did not express IL-17 (59, 62, 63). However, the co-
304 expression pattern of CXCR3 and CCR6 on Mtb- specific T cells present in BAL during latent
305 infection has not been studied. In the current study, we found that the majority of antigen
306 specific CD4 T cells co-expressed CXCR3 and CCR6 in BAL (**Figure 7**) and interestingly, unlike
307 in blood where IL-17 producing cells were absent (**Figure 5 and 6**), CXCR3+CCR6⁺ subsets in
308 macaque BAL were IL-17⁺ and IFN- γ +IL17⁺ co-producing cells.

309
310 In Mtb-infected macaques, CXCR3⁺ Th₁ cells have been shown to be efficient in localizing to
311 lung parenchyma during active TB (40) and in mouse models, these cells have been implicated
312 in containing salmonella within granulomas (64). However, the location of CXCR3⁺ CD4 cells in
313 the lung and their association with Mtb control in rhesus macaque lungs during LTBI have not
314 been previously described. Using immunofluorescence staining and quantification of
315 CXCR3+CD4⁺ cells in lung tissue sections from asymptomatic LTBI and Mtb active animals, we
316 found that CXCR3+CD4⁺ cells were located largely in granulomatous areas. Interestingly, the
317 density of CXCR3+CD4⁺ cells in the granuloma correlated inversely with bacterial load (**Figure**
318 **8**). Although we were unable to effectively stain for CCR6 in the lung, since majority of CCR6⁺
319 cells in the BAL also expressed CXCR3 (**Figure 7**), we conclude that CXCR3+CCR6⁺ T cells
320 subsets are likely to be associated with mycobacterial control in the lung.

321
322 Our studies clearly show that lung compartments of macaques that control Mtb infection are
323 populated with high levels of Mtb-specific Th₁ and Th₁₇ cells. These cells co-express CXCR3
324 and CCR6 and their presence correlates with lowered Mtb burdens in the lung. Our study also
325 highlights the unique value of the macaque model of LTBI for studying correlates of protective

326 immunity to TB at mucosal sites of infection. Future studies focusing on the cross-talk between
327 innate immunity and the development and maintenance of Mtb-specific T cell responses in the
328 BAL and lung will further extend our understanding of immune control of Mtb infection and
329 advance the development of better vaccines and immune therapeutics for TB.

330

331 **Methods**

332 **Infection of animals with Mtb**

333 The experimental design of these studies is described in **Figure 1**. Eight Indian-origin adult
334 rhesus macaques (*Macaca mulatta*) were exposed to ~10 CFU of Mtb CDC1551 as described
335 previously (19) resulting in all animals being infected, as assessed by the development of
336 positive TSTs and IGRAs. The animals were obtained from the Tulane National Primate
337 Research Center (TNPRC) breeding colony. Prior to the study, the animals were quarantined for
338 90 days and tested by both Tuberculin Skin Test (TST) and an NHP-specific IGRA
339 (PRIMAGAM, Prionics) (19) to ensure they were not previously exposed to Mtb infection. A
340 custom head-only dynamic inhalation system housed within a class III biological safety cabinet
341 was used for this purpose (19, 24, 27, 65). All animals tested positive by PRIMAGAM and TST
342 at 3 and 7 weeks post-infection. Six out of eight animals did not exhibit any signs or symptoms
343 of active TB and were considered to have LTBI when they remained asymptomatic for 15
344 weeks. All six animals remained asymptomatic for the duration of the 24-week study. Two out of
345 eight animals developed progressive primary TB disease and exhibited pyrexia, wasting, high
346 serum CRP, and other clinical signs of TB by seven weeks post-Mtb infection and were included
347 in a different study. All animal procedures were approved by the Institutional Animal Care and
348 Use Committee (IACUC) of Tulane University, New Orleans, LA, and were performed in strict
349 accordance with NIH guidelines. Mtb-infected animals were housed under BSL-3 conditions.

350

351 **Clinical procedures and sample collection**

352 Procedures for weekly complete blood counts (CBC), chemistry, chest radiographs (CXR) and
353 BAL at week 3 and every four weeks thereafter have been described previously (19, 24, 32, 66).
354 CXRs were scored by veterinary clinicians in a blinded fashion on a subjective scale of 0–4, with
355 a score of 0 denoting normal lung and a score of 4 denoting severe tuberculous pneumonia, as
356 previously described (19). Measurements of C-Reactive Protein (CRP), body weight and
357 temperature were performed as described earlier (19), at week -1 and at weeks 3, 7, 11, 15, 19
358 and 24. Peripheral blood and BAL samples were collected at 3, 7, 11, 15, 19 and 24 weeks after
359 Mtb infection. Peripheral blood mononuclear cells (PBMCs) were isolated from blood and
360 cryopreserved for subsequent antigen-specific flow cytometric assays. BAL samples were
361 obtained by bronchoscopy as previously described (19, 67), using two washes of 40 ml sterile
362 saline, and used for antigen-specific assays and to measure CFUs. Bacterial burden associated
363 with Mtb infection was determined in BAL and in lung at necropsy by plating BAL or
364 homogenized tissue sections as previously described (24, 27, 68, 69). Individual lung lobes
365 were cut into 2-mm thick slabs and stereologically selected for analysis which allows for
366 unbiased selection of lung tissue (70). Approximately 50% of the lung tissue was pooled by lung
367 lobe (n=5/animal), homogenized, serially diluted, and plated in quadruplicate for quantification of
368 bacterial load by CFU. Approximately 30% of the lung tissue was fixed for histological analysis
369 and the remaining tissue was fixed for use in immunohistochemistry and immunofluorescence
370 microscopy. The extent of morphometric lung pathology and the involvement of lung in
371 granulomatous lesions was also determined at necropsy. Animals were euthanized at 24 weeks
372 or upon signs of disease. Humane endpoints were predefined in the IACUC protocol and
373 applied as needed to reduce discomfort (27). All animals were routinely cared for according to
374 the guidelines prescribed by the National Institutes of Health Guide to Laboratory Animal Care.
375 All procedures were approved by IACUC, and the Institutional Biosafety Committee (IBC). The

376 TNPRC facilities are accredited by the American Association for Accreditation of Laboratory
377 Animal Care and licensed by the US Department of Agriculture.

378

379 **Antigen-specific assays and flow cytometry of PBMC and BAL samples**

380 Cell preparation tubes (CPT, BD Biosciences) were used for PBMC isolation (71) and PBMCs
381 were cryopreserved in 90% fetal FBS (Hyclone, South Logan, UT) and 10% dimethyl sulfoxide
382 (Sigma-Aldrich, St. Louis, MO, USA) until subsequent batch-testing by intracellular cytokine
383 staining (ICS) and flow cytometry. For processing of BAL, mononuclear cells were isolated by
384 passing through a 70- μ M nylon cell strainer (Becton Dickinson Discovery Labware, Bedford,
385 MA) followed by washing in complete media (RPMI-1640 containing 10% FBS, 2 mM glutamine,
386 100 IU/ml penicillin, and 100 μ g/ml streptomycin, Lonza, Walkersville, MD, USA). Isolated BAL
387 cells (approx. $1-2 \times 10^6$) were stimulated with CW antigen and ESAT-6/CFP-10 peptide pools for
388 2 hrs. at 37°C, 5% CO₂, Brefeldin-A was added and cells were further incubated for 4 hrs. at
389 37°C, 5% CO₂. The stimulated BAL cells were cryopreserved using 90% fetal FBS (Hyclone,
390 South Logan, UT) and 10% dimethyl sulfoxide (Sigma-Aldrich, St. Louis, MO, USA) and stored
391 in liquid nitrogen until subsequent batch processing for ICS staining and flow cytometry.

392

393 **Intracellular staining and flow cytometry**

394 Cryopreserved PBMCs were thawed (all time points for each animal were thawed on the same
395 day) and rested overnight at 37°C, 5% CO₂ in 10% complete RPMI. PBMCs (approx. $1-2 \times 10^6$)
396 were stimulated with Mtb CW antigens (10 μ g/ml; BEI Resources) or ESAT-6/CFP-10 peptide
397 pools (10 μ g/ml, 15-mers with 11 amino-acid overlap, Genemed Synthesis Inc., San Antonio,
398 TX, USA) for 2 hrs followed by the addition of Brefeldin A (10 μ g/ml) (BD Biosciences, San
399 Diego, CA, USA) after which the cells were further incubated for 16 hrs. ICS and flow cytometry

400 was performed as described below. Cryopreserved stimulated BAL cells were thawed (all time
401 points for each animal were thawed on the same day) and processed for ICS and flow
402 cytometry. Stimulated PBMCs and BAL cells were stained for dead cells using the LIVE/DEAD
403 Fixable Near-IR Dead Cell Stain (Life Technologies, OR) and then surface-stained with the
404 following antibodies: CD8-V500 (clone SK1), CCR6- BV711(clone 11A9), CD95-PETR (clone
405 DX2) from BD Biosciences, CD38-FITC (clone AT-1, Stemcell Technologies), CD28-PE-Cy7
406 (clone CD28.2) and CXCR3-BV605 (clone G025H7) from Biolegend. Cells were permeabilized
407 with Cytofix/Cytoperm Kit (BD Biosciences) and stained intracellularly with CD3-PerCP (clone
408 SP34-2), IFN- γ -Alexa Fluor 700 (clone B27) and IL-17-APC (clone MQ1-17H12) from BD
409 Biosciences and CD4 BV650 (clone OKT4, Biolegend). Cell were fixed with 1%
410 paraformaldehyde before acquisition in an LSR-II flow cytometer (BD Biosciences). Flow
411 cytometry data were analyzed using FlowJo software V10 (Tree Star Inc., San Carlos, CA,
412 USA).

413

414 **Histology and quantification of granulomatous areas in the lung**

415 Lung tissues were fixed in zinc-buffered formalin, processed routinely, and stained with
416 hematoxylin and eosin (H&E). The stained tissue sections were digitally scanned with a digital
417 slide scanner (Axio Scan.Z1, Carl Zeiss) and analyzed with computer software (Tissue
418 Classifier, HALO, Indica Labs). Annotation regions were drawn around each tissue section on
419 the slide. Annotated regions were then classified using an algorithm trained via a deep
420 convolutional network (HALO AI) to identify granulomas. All analyses were reviewed by a board-
421 certified veterinary pathologist to confirm their accuracy. Percentage of granuloma within tissue
422 sections was determined by dividing the granuloma area (mm^2) by the area of the annotated
423 regions (mm^2).

424 **Immunohistochemistry and confocal microscopy**

425 In addition to tissue sections from the six asymptomatic animals, we also included archived
426 formalin-fixed, paraffin-embedded lung tissues from animals with active TB that were reported in
427 our previous studies(24) and performed immunostaining and confocal microscopy as previously
428 described(27). Briefly, 5µm tissues sections were mounted on Superfrost Plus Microscope
429 slides, baked overnight at 56°C and passed through Xylene, graded ethanol, and double
430 distilled water to remove paraffin and rehydrate tissue sections. A microwave was used for heat
431 induced epitope retrieval (HIER). Slides were boiled for 20 minutes in a Tris based solution, pH
432 9 (Vector Labs H-3301), containing 0.01% Tween20. Slides were briefly rinsed in hot, distilled
433 water and transferred to a hot citrate based solution, pH 6.0 (Vector Labs H-3300) where they
434 were allowed to cool to room temperature. Once cool, slides were rinsed in tris buffered saline
435 (TBS) and placed in a black, humidifying chamber where they were incubated with Background
436 Punisher (Biocare Medical BP974H) for 10 minutes, washed with TBS containing 0.01%
437 TritonX100 (TBS-TX100) for 5 minutes, followed by a quick rinse in TBS before being returned
438 to the black chamber to be incubated with serum free protein block (Dako X0909) for 20
439 minutes. The slides were stained with primary antibodies against the following proteins: CD68
440 [1:20, mouse IgG1 (Dako, Carpinteria, CA)], CD163 [1:20, mouse IgG1 (Leica Biosystems
441 Buffalo Grove, IL)] CXCR3 [1:20 mouse IgG1 (BD Pharmingen, San Jose, CA)], CD4 [1:20,
442 rabbit (Abcam, Cambridge, MA)] and Dapi nuclear stain [1; 20,000 (Invitrogen, Carlsbad, CA)].
443 The above primary antibodies were detected with the following secondary antibodies from
444 Molecular Probes at a 1:1000 concentration derived from goat: Goat anti-mouse IgG1 488
445 (green), Goat anti-mouse IgG1 488 (green), Permanent Red, Goat anti-rabbit 647 (far red).
446 Imaging was performed with a Zeiss Axio Slide Scanner (Carl Zeiss, White Plains, NY), and the
447 images were analyzed with computer software (Hplex fluorescence, HALO, Indica Labs).

448
449

Quantification of CXCR3⁺ cells by immunofluorescence microscopy.

450 Tissue segmentation was first performed using pattern recognition software (Tissue Classifier,
451 HALO, Indica Labs). A random forest classifier was set at a resolution of 7 μ m/pixel and to detect
452 a minimum object size of 50 μ m². The classifier was then trained to detect the following tissue
453 classes by providing multiple examples of each tissue class: granuloma and non-granuloma.
454 Annotation regions were drawn around each piece of tissue on a slide and tissue segmentation
455 was performed on the entire piece of tissue (**Supplement Figure 3**). Computer software (Hplex
456 fluorescence, HALO, Indica Labs) was used to quantify the following phenotypes: CD4 cells,
457 CD4+CXCR3+ cells, CXCR3+ cells and CD68+/CD163+ macrophages. The software utilized
458 thresholding to both detect cells and set cut off values for expression of each marker/channel.
459 Thresholds were set by real time visual assessment of known positive and negative cells.
460 Analysis and quantification of cellular phenotypes were performed on each tissue segment as
461 defined above.

462

463 **Statistics**

464 Statistical analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA).
465 The specific tests used are indicated in each of the figure legends.

466 **Acknowledgements:**

467 We thank Toidi Adekambi for help with PBMC processing and optimization of flow cytometry
468 assays and Lakshmi Chennareddi for help with statistical analysis. We also thank present lab
469 members for their helpful suggestions and Jonathan Kevin Sia for comments on the manuscript.
470 We acknowledge the funding support from the following NIH grants: R01AI111943,
471 R01AI123047, R01AI134240, P51OD011133, and P51OD011104.

472

473 **Authors contributions:**

474 JR, DK and US conceived the studies. US, ANB, SRG, MQ, CI, XA, and RVB performed
475 experiments. US, JR, DK, ANB, CI, XA and VV performed data analyses. US, JR, DK and VV
476 wrote the manuscript.

477 **Disclosure/Conflict of Interest:** None

478 **References:**

- 479 1. . *Latent tuberculosis infection: updated and consolidated guidelines for programmatic*
480 *management*. Geneva; 2018.
- 481 2. Tait DR, Hatherill M, Van Der Meeren O, Ginsberg AM, Van Brakel E, Salaun B, et al.
482 Final Analysis of a Trial of M72/AS01E Vaccine to Prevent Tuberculosis. *N Engl J Med*.
483 2019.
- 484 3. Barry CE, 3rd, Boshoff HI, Dartois V, Dick T, Ehrt S, Flynn J, et al. The spectrum of
485 latent tuberculosis: rethinking the biology and intervention strategies. *Nat Rev Microbiol*.
486 2009;7(12):845-55.
- 487 4. Lin PL, Maiello P, Gideon HP, Coleman MT, Cadena AM, Rodgers MA, et al. PET CT
488 Identifies Reactivation Risk in Cynomolgus Macaques with Latent M. tuberculosis. *PLoS*
489 *Pathog*. 2016;12(7):e1005739.
- 490 5. Lin PL, and Flynn JL. The End of the Binary Era: Revisiting the Spectrum of
491 Tuberculosis. *J Immunol*. 2018;201(9):2541-8.
- 492 6. Flynn JL, Chan J, Triebold KJ, Dalton DK, Stewart TA, and Bloom BR. An essential role
493 for interferon gamma in resistance to Mycobacterium tuberculosis infection. *J Exp Med*.
494 1993;178(6):2249-54.
- 495 7. Green AM, DiFazio R, and Flynn JL. IFN-gamma from CD4 T Cells Is Essential for Host
496 Survival and Enhances CD8 T Cell Function during Mycobacterium tuberculosis
497 Infection. *J Immunol*. 2013;190(1):270-7.
- 498 8. Zhao HM, Du R, Li CL, Ji P, Li HC, Wu K, et al. Differential T cell responses against
499 DosR-associated antigen Rv2028c in BCG-vaccinated populations with tuberculosis
500 infection. *J Infect*. 2019;78(4):275-80.
- 501 9. Lu LL, Smith MT, Yu KKQ, Luedemann C, Suscovich TJ, Grace PS, et al. IFN-gamma-
502 independent immune markers of Mycobacterium tuberculosis exposure. *Nat Med*.
503 2019;25(6):977-87.
- 504 10. Essone PN, Leboueny M, Maloupazoa Siawaya AC, Alame-Emane AK, Aboumegone
505 Biyogo OC, Dapnet Tadatsin PH, et al. M. tuberculosis infection and antigen specific
506 cytokine response in healthcare workers frequently exposed to tuberculosis. *Sci Rep*.
507 2019;9(1):8201.
- 508 11. Schuck SD, Mueller H, Kunitz F, Neher A, Hoffmann H, Franken KL, et al. Identification
509 of T-cell antigens specific for latent mycobacterium tuberculosis infection. *Plos One*.
510 2009;4(5):e5590.
- 511 12. Barham MS, Abrahams DA, Khayumbi J, Ongalo J, Tonui J, Campbell A, et al. HIV
512 Infection Is Associated With Downregulation of BTLA Expression on Mycobacterium

- 513 tuberculosis-Specific CD4T Cells in Active Tuberculosis Disease. *Frontiers in*
514 *Immunology*. 2019;10.
- 515 13. Chiacchio T, Petruccioli E, Vanini V, Butera O, Cuzzi G, Petrone L, et al. Higher
516 frequency of T-cell response to M. tuberculosis latency antigen Rv2628 at the site of
517 active tuberculosis disease than in peripheral blood. *PLoS One*. 2011;6(11):e27539.
- 518 14. Jarvela JR, Tuscano L, Lee H, and Silver RF. Pulmonary responses to pathogen-specific
519 antigens in latent Mycobacterium tuberculosis infection. *Tuberculosis (Edinb)*.
520 2016;96:158-64.
- 521 15. Jarvela J, Moyer M, Leahy P, Bonfield T, Fletcher D, Mkono WN, et al. Mycobacterium
522 tuberculosis-Induced Bronchoalveolar Lavage Gene Expression Signature in Latent
523 Tuberculosis Infection Is Dominated by Pleiotropic Effects of CD4(+) T Cell-Dependent
524 IFN-gamma Production despite the Presence of Polyfunctional T Cells within the
525 Airways. *J Immunol*. 2019;203(8):2194-209.
- 526 16. Capuano SV, Croix DA, Pawar S, Zinovik A, Myers A, Lin PL, et al. Experimental
527 Mycobacterium tuberculosis infection of cynomolgus Macaques closely resembles the
528 various manifestations of human M-tuberculosis infection. *Infect Immun*.
529 2003;71(10):5831-44.
- 530 17. Walsh GP, Tan EV, delaCruz EC, Abalos RM, Villahermosa LG, Young LJ, et al. The
531 Philippine cynomolgus monkey (*Macaca fascicularis*) provides a new nonhuman primate
532 model of tuberculosis that resembles human disease. *Nat Med*. 1996;2(4):430-6.
- 533 18. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative
534 Comparison of Active and Latent Tuberculosis in the Cynomolgus Macaque Model.
535 *Infect Immun*. 2009;77(10):4631-42.
- 536 19. Mehra S, Golden NA, Dutta NK, Midkiff CC, Alvarez X, Doyle LA, et al. Reactivation of
537 latent tuberculosis in rhesus macaques by coinfection with simian immunodeficiency
538 virus. *Journal of medical primatology*. 2011;40(4):233-43.
- 539 20. Foreman TW, Bucsan AN, Mehra S, Peloquin C, Doyle LA, Russell-Lodrigue K, et al.
540 Isoniazid and Rifapentine Treatment Eradicates Persistent Mycobacterium tuberculosis
541 in Macaques. *American journal of respiratory and critical care medicine*. 2019.
- 542 21. Bucsan AN, Rout N, Foreman TW, Khader SA, Rengarajan J, and Kaushal D. Mucosal-
543 activated invariant T cells do not exhibit significant lung recruitment and proliferation
544 profiles in macaques in response to infection with Mycobacterium tuberculosis
545 CDC1551. *Tuberculosis (Edinb)*. 2019;116S:S11-S8.
- 546 22. Bucsan AN, Chatterjee A, Singh DK, Foreman TW, Lee TH, Threeton B, et al.
547 Mechanisms of reactivation of latent tuberculosis infection due to SIV coinfection. *J Clin*
548 *Invest*. 2019.
- 549 23. Mehra S, Foreman TW, Didier PJ, Ahsan MH, Hudock TA, Kisse R, et al. The DosR
550 Regulon Modulates Adaptive Immunity and is Essential for M. tuberculosis Persistence.
551 *American journal of respiratory and critical care medicine*. 2015.
- 552 24. Foreman TW, Mehra S, LoBato DN, Malek A, Alvarez X, Golden NA, et al. CD4+ T-cell-
553 independent mechanisms suppress reactivation of latent tuberculosis in a macaque
554 model of HIV coinfection. *Proc Natl Acad Sci U S A*. 2016;113(38):E5636-44.
- 555 25. Sallusto F, Lenig D, Forster R, Lipp M, and Lanzavecchia A. Two subsets of memory T
556 lymphocytes with distinct homing potentials and effector functions. *Nature*.
557 1999;401(6754):708-12.
- 558 26. Lindenstrom T, Agger EM, Korsholm KS, Darrah PA, Aagaard C, Seder RA, et al.
559 Tuberculosis subunit vaccination provides long-term protective immunity characterized
560 by multifunctional CD4 memory T cells. *J Immunol*. 2009;182(12):8047-55.
- 561 27. Kaushal D, Foreman TW, Gautam US, Alvarez X, Adekambi T, Rangel-Moreno J, et al.
562 Mucosal vaccination with attenuated Mycobacterium tuberculosis induces strong central
563 memory responses and protects against tuberculosis. *Nat Commun*. 2015;6:8533.

- 564 28. Dijkman K, Sombroek CC, Vervenne RAW, Hofman SO, Boot C, Remarque EJ, et al.
565 Prevention of tuberculosis infection and disease by local BCG in repeatedly exposed
566 rhesus macaques. *Nat Med.* 2019;25(2):255-+.
- 567 29. Groom JR, and Luster AD. CXCR3 in T cell function. *Exp Cell Res.* 2011;317(5):620-31.
- 568 30. Kim CH. Migration and function of Th17 cells. *Inflamm Allergy Drug Targets.*
569 2009;8(3):221-8.
- 570 31. Ito T, Carson WF, Cavassani KA, Connett JM, and Kunkel SL. CCR6 as a mediator of
571 immunity in the lung and gut. *Experimental Cell Research.* 2011;317(5):613-9.
- 572 32. Kuroda MJ, Sugimoto C, Cai Y, Merino KM, Mehra S, Arainga M, et al. High Turnover of
573 Tissue Macrophages Contributes to Tuberculosis Reactivation in Simian
574 Immunodeficiency Virus-Infected Rhesus Macaques. *The Journal of infectious diseases.*
575 2018;217(12):1865-74.
- 576 33. Lin PL, Rodgers M, Smith L, Bigbee M, Myers A, Bigbee C, et al. Quantitative
577 comparison of active and latent tuberculosis in the cynomolgus macaque model. *Infect*
578 *Immun.* 2009;77(10):4631-42.
- 579 34. Mehra S, Alvarez X, Didier PJ, Doyle LA, Blanchard JL, Lackner AA, et al. Granuloma
580 correlates of protection against tuberculosis and mechanisms of immune modulation by
581 *Mycobacterium tuberculosis*. *The Journal of infectious diseases.* 2013;207(7):1115-27.
- 582 35. Lin PL, Pawar S, Myers A, Pegu A, Fuhrman C, Reinhart TA, et al. Early events in
583 *Mycobacterium tuberculosis* infection in cynomolgus macaques. *Infect Immun.*
584 2006;74(7):3790-803.
- 585 36. Foreman TW, Mehra S, Lackner AA, and Kaushal D. Translational Research in the
586 Nonhuman Primate Model of Tuberculosis. *ILAR J.* 2017;58(2):151-9.
- 587 37. Bucsan AN, Mehra S, Khader SA, and Kaushal D. The current state of animal models
588 and genomic approaches towards identifying and validating molecular determinants of
589 *Mycobacterium tuberculosis* infection and tuberculosis disease. *Pathogens and disease.*
590 2019;77(4).
- 591 38. Shen L, Frencher J, Huang D, Wang W, Yang E, Chen CY, et al. Immunization of
592 Vgamma2Vdelta2 T cells programs sustained effector memory responses that control
593 tuberculosis in nonhuman primates. *Proceedings of the National Academy of Sciences*
594 *of the United States of America.* 2019;116(13):6371-8.
- 595 39. Lin PL, Ford CB, Coleman MT, Myers AJ, Gawande R, Ioerger T, et al. Sterilization of
596 granulomas is common in active and latent tuberculosis despite within-host variability in
597 bacterial killing. *Nat Med.* 2014;20(1):75-9.
- 598 40. Kauffman KD, Sallin MA, Sakai S, Kamenyeva O, Kabat J, Weiner D, et al. Defective
599 positioning in granulomas but not lung-homing limits CD4 T-cell interactions with
600 *Mycobacterium tuberculosis*-infected macrophages in rhesus macaques. *Mucosal*
601 *Immunol.* 2018;11(2):462-73.
- 602 41. Diedrich CR, Gideon HP, Rutledge T, Baranowski TM, Maiello P, Myers AJ, et al.
603 CD4CD8 Double Positive T cell responses during *Mycobacterium tuberculosis* infection
604 in cynomolgus macaques. *J Med Primatol.* 2019;48(2):82-9.
- 605 42. Silver RF, Myers AJ, Jarvela J, Flynn J, Rutledge T, Bonfield T, et al. Diversity of Human
606 and Macaque Airway Immune Cells at Baseline and during Tuberculosis Infection. *Am J*
607 *Resp Cell Mol.* 2016;55(6):899-908.
- 608 43. Darrah PA, Bolton DL, Lackner AA, Kaushal D, Aye PP, Mehra S, et al. Aerosol
609 vaccination with AERAS-402 elicits robust cellular immune responses in the lungs of
610 rhesus macaques but fails to protect against high-dose *Mycobacterium tuberculosis*
611 challenge. *J Immunol.* 2014;193(4):1799-811.
- 612 44. Darrah PA, DiFazio RM, Maiello P, Gideon HP, Myers AJ, Rodgers MA, et al. Boosting
613 BCG with proteins or rAd5 does not enhance protection against tuberculosis in rhesus
614 macaques. *Npj Vaccines.* 2019;4.

- 615 45. Darrah PA, Zeppa JJ, Maiello P, Hackney JA, Wadsworth MH, 2nd, Hughes TK, et al.
616 Prevention of tuberculosis in macaques after intravenous BCG immunization. *Nature*.
617 2020;577(7788):95-102.
- 618 46. Cooper AM, Dalton DK, Stewart TA, Griffin JP, Russell DG, and Orme IM. Disseminated
619 tuberculosis in interferon gamma gene-disrupted mice. *J Exp Med*. 1993;178(6):2243-7.
- 620 47. Lin PL, Rutledge T, Green AM, Bigbee M, Fuhrman C, Klein E, et al. CD4 T Cell
621 Depletion Exacerbates Acute Mycobacterium tuberculosis While Reactivation of Latent
622 Infection Is Dependent on Severity of Tissue Depletion in Cynomolgus Macaques. *Aids*
623 *Res Hum Retrov*. 2012;28(12):1693-702.
- 624 48. Yao S, Huang D, Chen CY, Halliday L, Wang RC, and Chen ZW. CD4+ T cells contain
625 early extrapulmonary tuberculosis (TB) dissemination and rapid TB progression and
626 sustain multi-effector functions of CD8+ T and CD3- lymphocytes: mechanisms of CD4+
627 T cell immunity. *J Immunol*. 2014;192(5):2120-32.
- 628 49. Iwanaga N, and Kolls JK. Updates on T helper type 17 immunity in respiratory disease.
629 *Immunology*. 2019;156(1):3-8.
- 630 50. Van Dis E, Sogi KM, Rae CS, Sivick KE, Surh NH, Leong ML, et al. STING-Activating
631 Adjuvants Elicit a Th17 Immune Response and Protect against Mycobacterium
632 tuberculosis Infection. *Cell Rep*. 2018;23(5):1435-47.
- 633 51. Gallegos AM, van Heijst JWJ, Samstein M, Su XD, Pamer EG, and Glickman MS. A
634 Gamma Interferon Independent Mechanism of CD4 T Cell Mediated Control of M.
635 tuberculosis Infection in vivo. *Plos Pathogens*. 2011;7(5).
- 636 52. Gopal R, Monin L, Slight S, Uche U, Blanchard E, Fallert Junecko BA, et al. Unexpected
637 role for IL-17 in protective immunity against hypervirulent Mycobacterium tuberculosis
638 HN878 infection. *PLoS pathogens*. 2014;10(5):e1004099.
- 639 53. Wareham AS, Tree JA, Marsh PD, Butcher PD, Dennis M, and Sharpe SA. Evidence for
640 a role for interleukin-17, Th17 cells and iron homeostasis in protective immunity against
641 tuberculosis in cynomolgus macaques. *Plos One*. 2014;9(2):e88149.
- 642 54. Gideon HP, Phuah J, Myers AJ, Bryson BD, Rodgers MA, Coleman MT, et al. Variability
643 in Tuberculosis Granuloma T Cell Responses Exists, but a Balance of Pro- and Anti-
644 inflammatory Cytokines Is Associated with Sterilization. *Plos Pathogens*. 2015;11(1).
- 645 55. Pitt JM, Stavropoulos E, Redford PS, Beebe AM, Bancroft GJ, Young DB, et al.
646 Blockade of IL-10 signaling during bacillus Calmette-Guerin vaccination enhances and
647 sustains Th1, Th17, and innate lymphoid IFN-gamma and IL-17 responses and
648 increases protection to Mycobacterium tuberculosis infection. *J Immunol*.
649 2012;189(8):4079-87.
- 650 56. Sia JK, Bizzell E, Madan-Lala R, and Rengarajan J. Engaging the CD40-CD40L
651 pathway augments T-helper cell responses and improves control of Mycobacterium
652 tuberculosis infection. *PLoS Pathog*. 2017;13(8):e1006530.
- 653 57. Orme IM. Pillars Article: The Kinetics of Emergence and Loss of Mediator T
654 Lymphocytes Acquired in Response to Infection with Mycobacterium Tuberculosis. *J*
655 *Immunol*. 1987. 138: 293-298. *J Immunol*. 2017;199(3):833-8.
- 656 58. Bluestone JA, Mackay CR, O'Shea JJ, and Stockinger B. The functional plasticity of T
657 cell subsets. *Nat Rev Immunol*. 2009;9(11):811-6.
- 658 59. Acosta-Rodriguez EV, Rivino L, Geginat J, Jarrossay D, Gattorno M, Lanzavecchia A, et
659 al. Surface phenotype and antigenic specificity of human interleukin 17-producing T
660 helper memory cells. *Nat Immunol*. 2007;8(6):639-46.
- 661 60. Ramesh R, Kozhaya L, McKevitt K, Djuretic IM, Carlson TJ, Quintero MA, et al. Pro-
662 inflammatory human Th17 cells selectively express P-glycoprotein and are refractory to
663 glucocorticoids. *J Exp Med*. 2014;211(1):89-104.
- 664 61. Silveira-Mattos PS, Narendran G, Akrami K, Fukutani KF, Anbalagan S, Nayak K, et al.
665 Differential expression of CXCR3 and CCR6 on CD4(+) T-lymphocytes with distinct

- 666 memory phenotypes characterizes tuberculosis-associated immune reconstitution
667 inflammatory syndrome. *Sci Rep-Uk*. 2019;9.
- 668 62. Lindestam Arlehamn CS, Gerasimova A, Mele F, Henderson R, Swann J, Greenbaum
669 JA, et al. Memory T cells in latent Mycobacterium tuberculosis infection are directed
670 against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset.
671 *PLoS Pathog*. 2013;9(1):e1003130.
- 672 63. Arlehamn CL, Seumois G, Gerasimova A, Huang C, Fu Z, Yue X, et al. Transcriptional
673 profile of tuberculosis antigen-specific T cells reveals novel multifunctional features. *J*
674 *Immunol*. 2014;193(6):2931-40.
- 675 64. Goldberg MF, Roeske EK, Ward LN, Pengo T, Dileepan T, Kotov DI, et al. Salmonella
676 Persist in Activated Macrophages in T Cell-Sparse Granulomas but Are Contained by
677 Surrounding CXCR3 Ligand-Positioned Th1 Cells. *Immunity*. 2018;49(6):1090-102 e7.
- 678 65. Hartings JM, and Roy CJ. The automated bioaerosol exposure system: preclinical
679 platform development and a respiratory dosimetry application with nonhuman primates.
680 *J Pharmacol Toxicol Methods*. 2004;49(1):39-55.
- 681 66. Corleis B, Bucsan AN, Deruaz M, Vrbanac VD, Lisanti-Park AC, Gates SJ, et al. HIV-1
682 and SIV Infection Are Associated with Early Loss of Lung Interstitial CD4+ T Cells and
683 Dissemination of Pulmonary Tuberculosis. *Cell reports*. 2019;26(6):1409-18 e5.
- 684 67. Mehra S, Foreman TW, Didier PJ, Ahsan MH, Hudock TA, Kisse R, et al. The DosR
685 Regulon Modulates Adaptive Immunity and Is Essential for Mycobacterium tuberculosis
686 Persistence. *Am J Resp Crit Care*. 2015;191(10):1185-96.
- 687 68. Kaushal D, Mehra S, Didier PJ, and Lackner AA. The non-human primate model of
688 tuberculosis. *Journal of medical primatology*. 2012;41(3):191-201.
- 689 69. Dutta NK, McLachlan J, Mehra S, and Kaushal D. Humoral and lung immune responses
690 to Mycobacterium tuberculosis infection in a primate model of protection(). *Trials*
691 *Vaccinol*. 2014;3:47-51.
- 692 70. Luciw PA, Oslund KL, Yang XW, Adamson L, Ravindran R, Canfield DR, et al.
693 Stereological analysis of bacterial load and lung lesions in nonhuman primates (rhesus
694 macaques) experimentally infected with Mycobacterium tuberculosis. *Am J Physiol Lung*
695 *Cell Mol Physiol*. 2011;301(5):L731-8.
- 696 71. Adekambi T, Ibegbu CC, Cagle S, Kalokhe AS, Wang YF, Hu Y, et al. Biomarkers on
697 patient T cells diagnose active tuberculosis and monitor treatment response. *J Clin*
698 *Invest*. 2015;125(5):1827-38.
- 699

701 **Figure Legends**

702

703 **Figure 1: Experimental design and clinical characteristics of asymptomatic rhesus**

704 **macaques with LTBI.** (A) Eight Rhesus macaques were infected by low dose aerosol route

705 with Mtb CDC1551 at week 0 and infection was confirmed at 3 and 7 weeks by TST and IGRA

706 tests. Six macaques were defined as LTBI based absence of clinical signs and symptoms of

707 disease and a negative chest X-ray up to week 15. Six animals remained asymptomatic and

708 were longitudinally followed up until week 24. PBMC and BAL were collected at indicated time

709 points and lung tissues were collected at necropsy. Each colored symbol represents an animal.

710 (B) CRP levels were measured prior to infection (week -1) and at indicated time points post-

711 infection for n=6 animals. (C) Mtb burden (CFU) in BAL measured at indicated weeks (D) Mtb

712 burden (CFU) in lung at necropsy (CFU per gram of tissue plated). (E) Percentage of granuloma

713 in the lung tissue, determined by dividing the granulomatous area (mm²) by the area of the

714 annotated regions (mm²) classified using an algorithm trained via a deep convolutional network

715 (HALO AI).

716

717 **Figure 2: Frequencies of CD4 and CD8 T cells in BAL and PBMC.**

718 Red and Blue circles indicate BAL and PBMC respectively. (A) Higher frequencies of CD4 T

719 cells in PBMC compared to BAL at all indicated time points ($p=0.01$). (B) Higher frequencies of

720 CD8 T cells in BAL compared to PBMC at all indicated time points ($p=0.01$). Each data point

721 represents an animal and horizontal lines indicate the mean with SEM. Wilcoxon matched-pairs

722 signed rank test was used to compare the frequencies of CD4 and CD8 T cells between BAL

723 and PBMC.

724

725

726 **Figure 3: Kinetics of Mtb-specific CD4 and CD8 T cells producing IFN- γ in PBMC and**
727 **BAL.**

728 PBMCs (blue circles) and BAL (red circles) were stimulated with CW and ESAT-6/CFP-10
729 peptide pools and IFN- γ production by CD4 and CD8 T cells were assessed by ICS and flow
730 cytometry. IFN- γ producing CD4 (A & B) and CD8 (C & D) T cells at indicated time points
731 (weeks) post-Mtb infection in macaques (n=6) with LTBI. CW-specific CD4 (A) and CD8 (C) T
732 cell frequencies and ESAT-6/CFP-10-specific CD4 (B) and CD8 (D) T cell frequencies in PBMC
733 and BAL are shown. Horizontal lines indicate the mean with SEM. Wilcoxon matched-pairs
734 signed rank test was used to compare the frequencies of IFN- γ producing CD4 and CD8 T cells
735 between BAL and PBMC.

736

737

738 **Figure 4: Memory phenotypes of Mtb-specific CD4 and CD8 T cells in PBMC and BAL.**

739 (A, B) Representative flow plots of IFN- γ + CD4 and CD8 T cells expressing CD28 and CD95 in
740 PBMC and in BAL respectively following CW and ESAT-6/CFP-10 stimulation. (C, D)
741 Frequencies of CW-and ESAT-6/CFP-10-specific memory (CD28+CD95+, Purple) and effector
742 (CD28-CD95+, Orange) CD4 and CD8 T cells in PBMC and BAL at week 7 post- Mtb infection.
743 (E-H) Kinetics of CW and ESAT-6/CFP-10 specific memory (Orange bar) and effector (purple
744 bar) CD4 and CD8 T cells in PBMC and BAL. Horizontal lines indicate the mean with SEM. ** p
745 <0.01, * p <0.05 (paired T-test, 2-tailed).

746

747 **Figure 5: Kinetics of Mtb-specific CD4 and CD8 T cells producing IL-17 in PBMC and**
748 **BAL.**

749 PBMCs (blue circles) and BAL (red circles) samples were stimulated with CW (A & C) and
750 ESAT-6/CFP-10 peptide pools (B & D) and IL-17 production by CD4 (A & B) and CD8 (C & D) T

751 cells assessed by ICS and flow cytometry at the time points indicated (weeks) post-Mtb
752 infection. Horizontal lines indicate the mean with SEM.

753

754 **Figure 6: Higher frequencies of IL-17+ and IFN- γ IL-17 double positive Mtb-specific CD4 T**
755 **cells in the BAL compared to PBMCs.**

756 (A) Representative flow plots of week 7 PBMC and BAL that are either non-stimulated (NS) or
757 stimulated with CW- and ESAT-6/CFP-10 peptide pools. CD4 and CD8 T cells expressing IFN- γ
758 and IL-17 were assessed by ICS and flow cytometry. Frequencies of single-positive IFN- γ (B)
759 single-positive IL-17 (C) and IFN- γ /IL-17 double-positive (D) CD4 and CD8 T cells in PBMC
760 (Blue) and BAL (red). * $p < 0.05$ using a nonparametric Mann-Whitney test. Data are
761 represented as mean with SEM.

762

763 **Figure 7: Mtb-specific CD4+ T cells producing IFN- γ and IL-17 co- expressed CXCR3 and**
764 **CCR6.**

765 Frequencies of ESAT-6/CFP-10-specific IFN- γ + (A) and IL-17+ (B) CD4 T cells in the BAL that
766 expressed either CXCR3 (brown bar) or CCR6 (black bar). Majority of IFN- γ and IL-17
767 producing CD4 T cells expressed CXCR3 at all time points. Mtb-specific IFN- γ + (C & D) and IL-
768 17+ (E & F) CD4 T cells in BAL co-expressing CXCR3 and CCR6 (Blue bar) were significantly
769 higher compared to CXCR3+CCR6- (orange bar) and CXCR3-CCR6+ (Green bar) subsets.
770 IFN- γ /IL-17 double-positive cells were also predominantly CXCR3+CCR6+ (G & H). Horizontal
771 lines indicate mean \pm SEM. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$ (paired T test, 2-tailed).

772 **Figure 8: CXCR3+CD4 T cells were predominant in the granulomatous region of the lung.**

773 (A) Representative immunohistochemistry staining of lung sections from macaques with LTBI
774 and active TB. Macrophages (green), CD4 and CXCR3 (red) and nuclei (DAPI). Left panel
775 shows low (5mm) magnification images of lung sections from macaques with asymptomatic

776 LTBI and active TB (N=3, each group). Middle and right panels show images of the granuloma
777 (500 μ m) and non-granuloma (100 μ m) areas of lung sections, respectively (B) Densities of
778 CD4+CXCR3+ cells in lung tissue of animals with LTBI and active TB in the lung (total) and in
779 granulomatous (G) and non-granulomatous (NG) areas of the lung. Density of CD4+CXCR3+
780 cells was measured by dividing the number of CD4+CXCR3+ cells quantified by the area in
781 mm² calculated from H& E stained tissue using algorithms trained via a deep convolutional
782 network (HALO, Indica Labs). Comparison within and between groups were performed using
783 nonparametric Wilcoxon matched-pairs signed rank test and Mann-Whitney test respectively.
784 Horizontal lines indicate mean \pm SEM. * $p < 0.05$, ** $p < 0.01$. (C) Lung bacterial burden negatively
785 ($p = 0.002$) correlated with the density of lung CXCR3+ CD4 T cells from animals with LTBI and
786 active TB. Correlation was performed using non-parametric Spearman correlation method.

787

788 **Supplementary Figure 1:** A) Weight and B) Temperature did not change pre- and post-Mtb
789 infection and until week 24 indicating asymptomatic latent infection in rhesus macaques.

790

791

792 **Supplementary Figure 2:** Frequencies of CW- and ESAT-6/CFP-10 specific CD4 and CD8 T
793 cells producing IFN- γ (A & B) and IL-17 (B & D) in PBMC at baseline and week 3.

794

795 **Supplementary Figure 3: Representative image showing granuloma and non-granuloma**

796 **region classification:** Tissue segmentation performed using pattern recognition software

797 (Tissue Classifier, HALO, Indica Labs). A random forest classifier was set at a resolution of

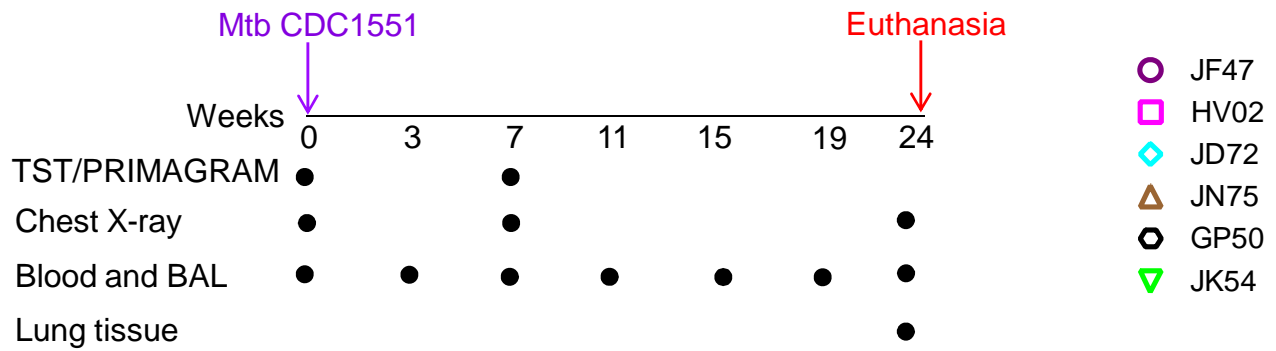
798 7 μ m/pixel and to detect a minimum object size of 50 μ m². The classifier was then trained to

799 detect the following tissue classes by providing multiple examples of each tissue class:

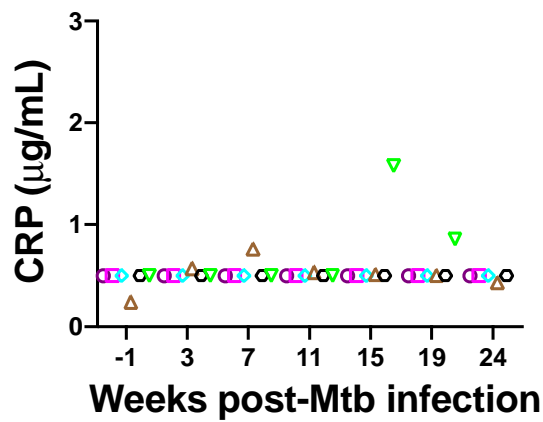
800 granuloma (area in brown and blue) and non-granuloma (area in green). Annotation regions

801 were drawn around each piece of tissue on a slide and tissue segmentation was performed on
802 the entire piece of tissue
803

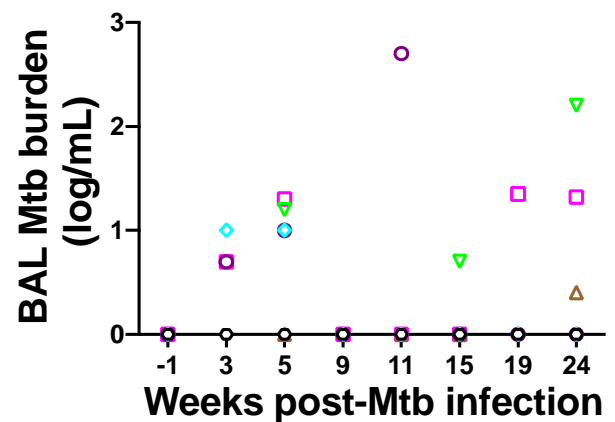
A



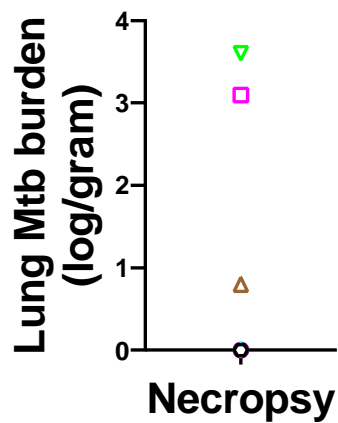
B



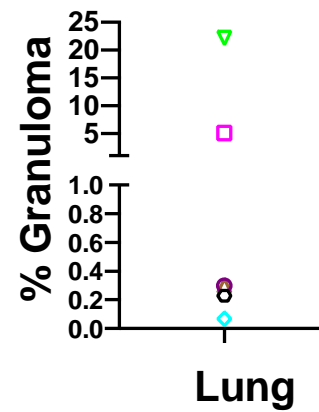
C

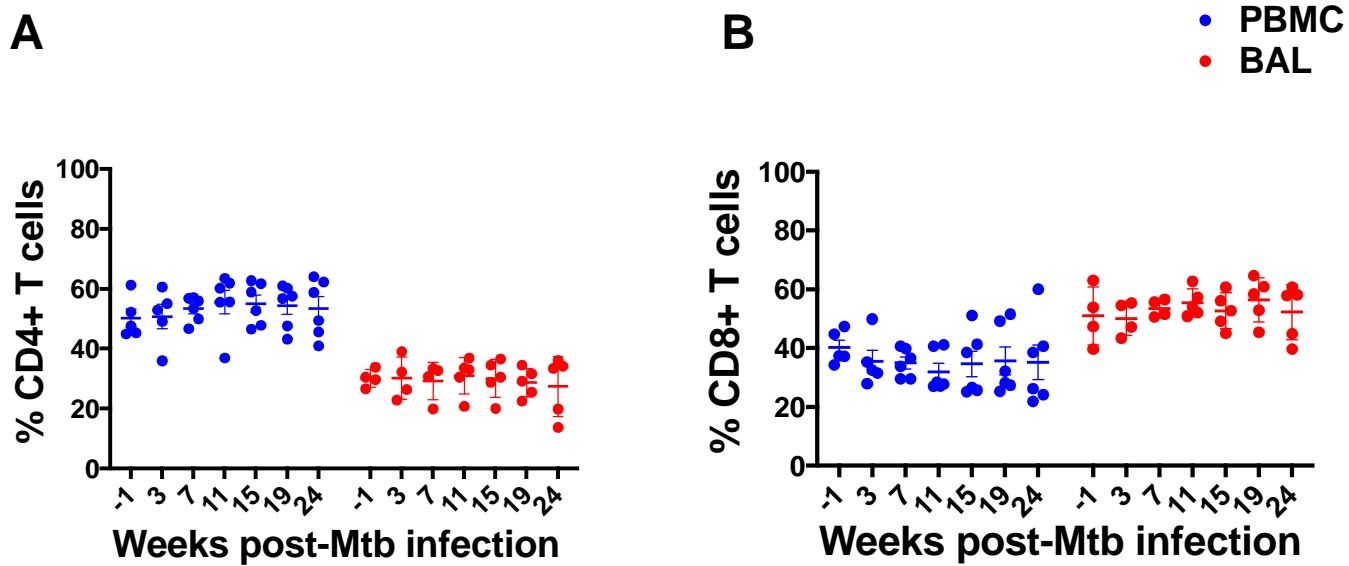


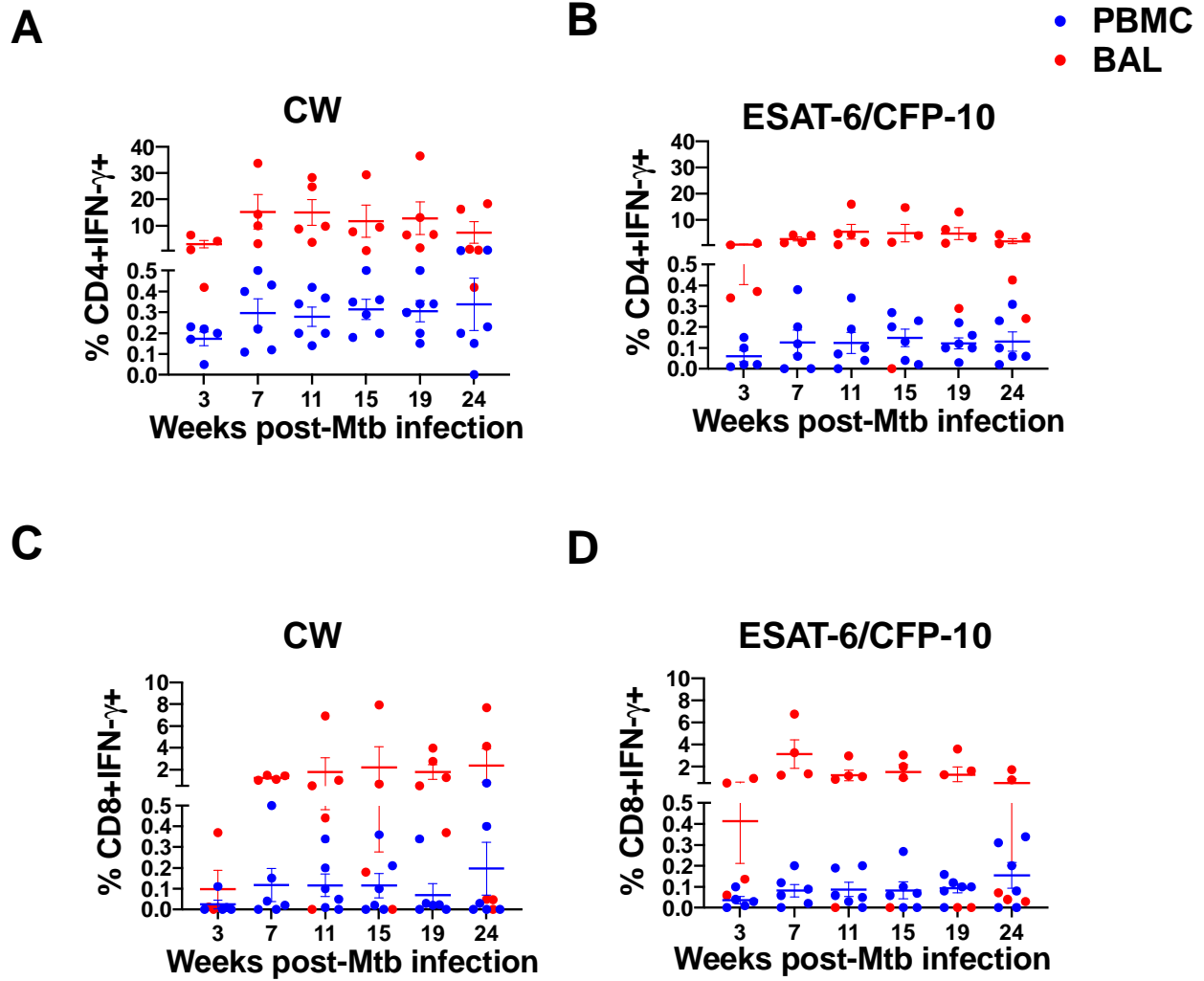
D

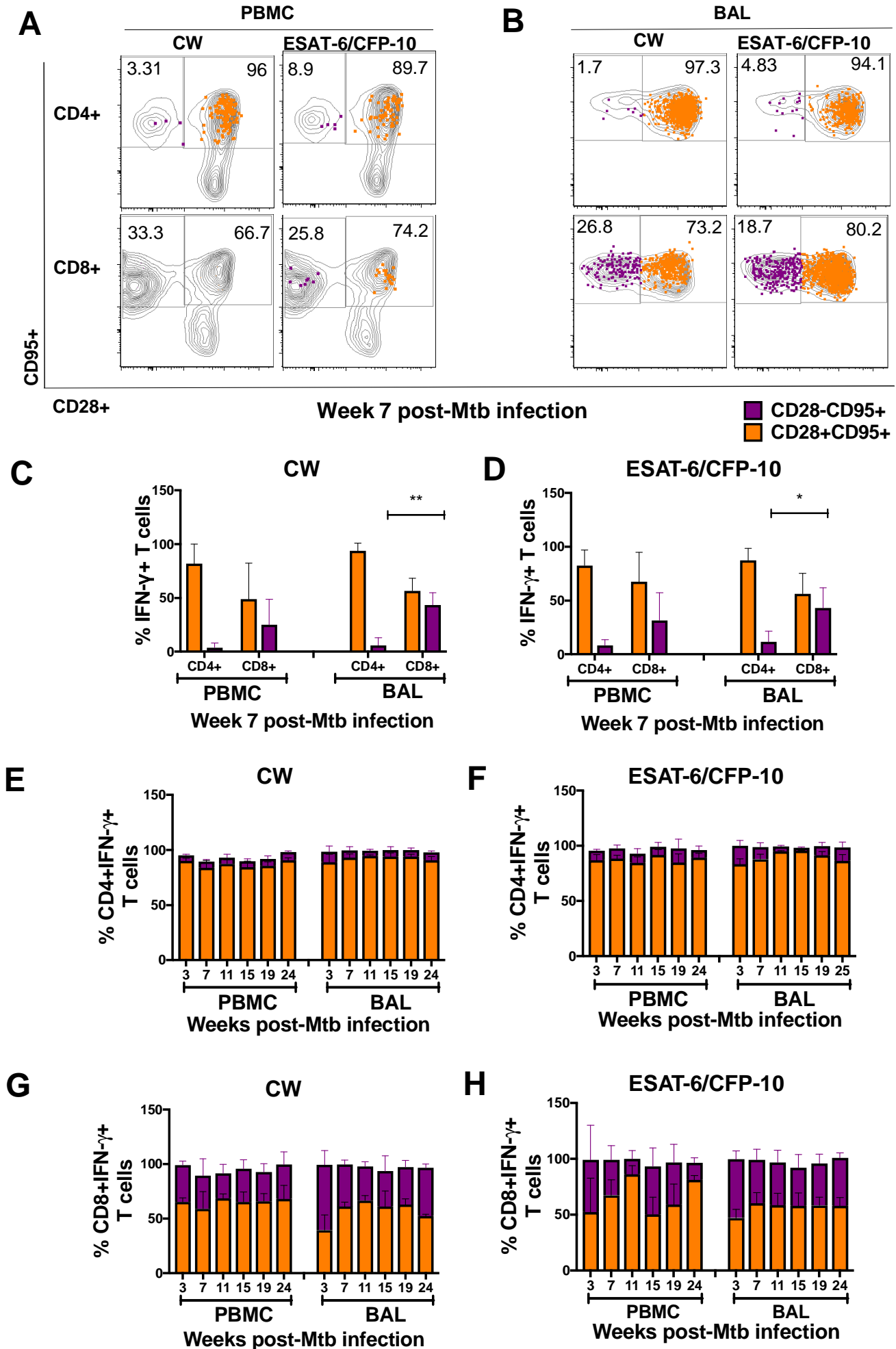


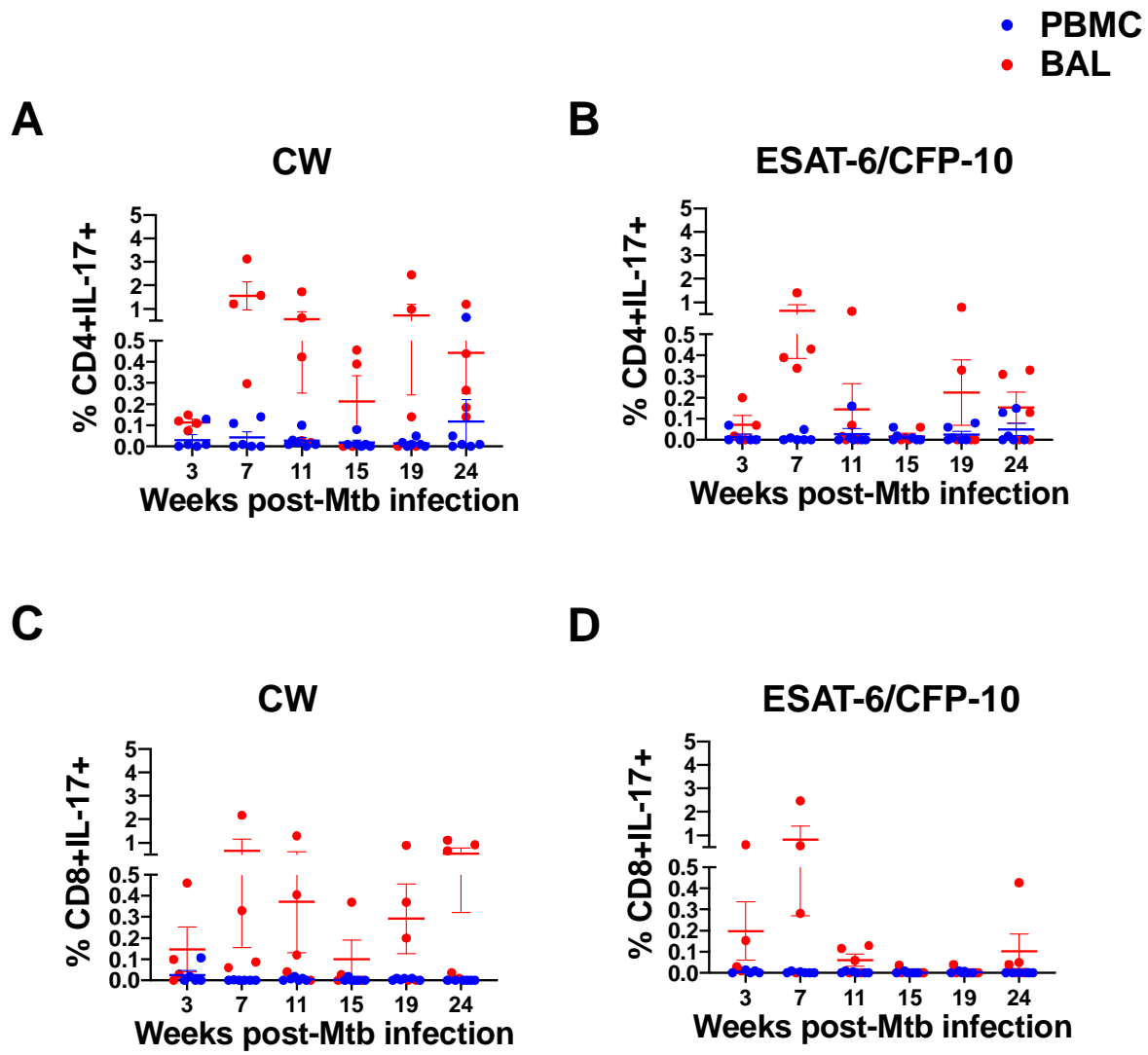
E

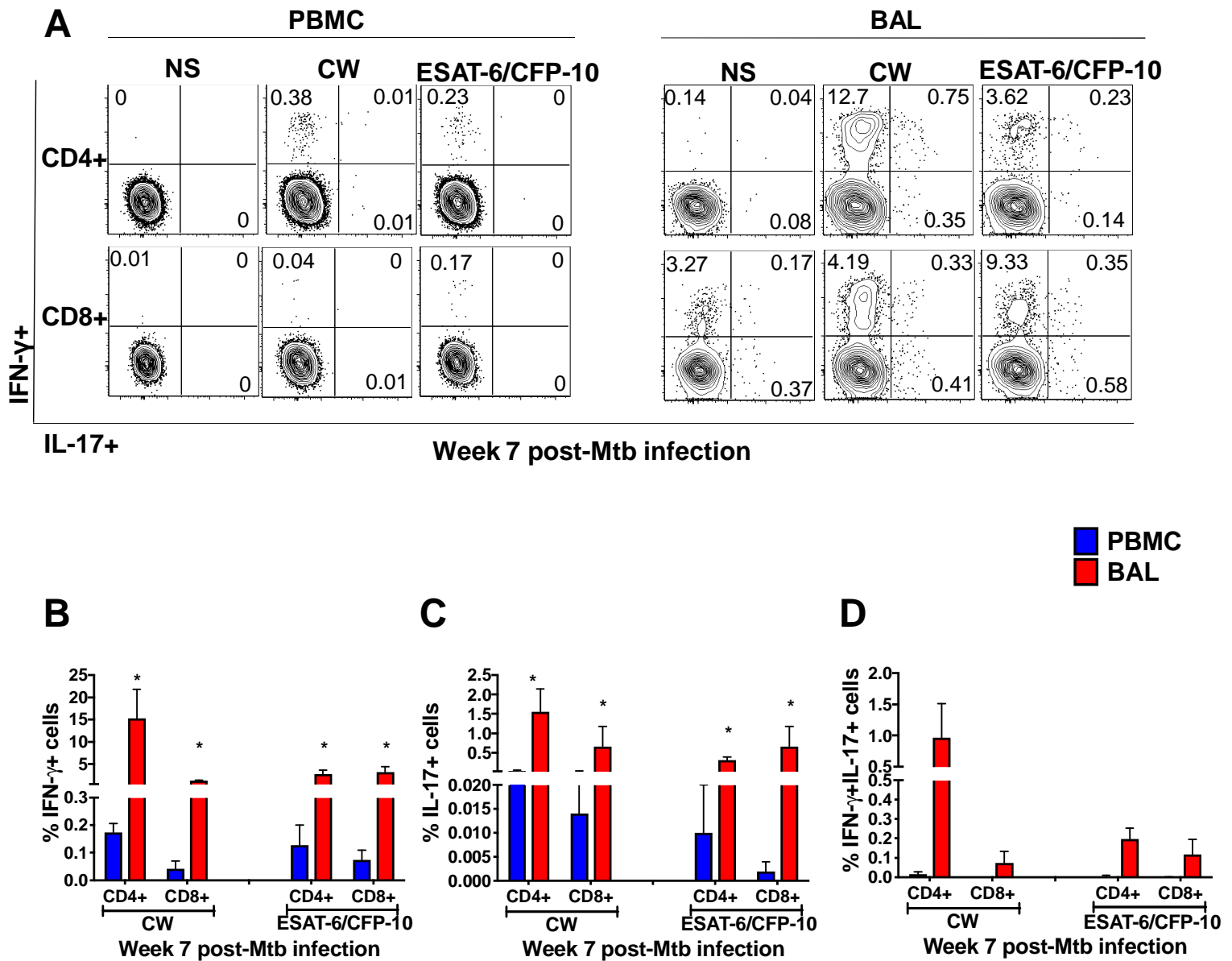






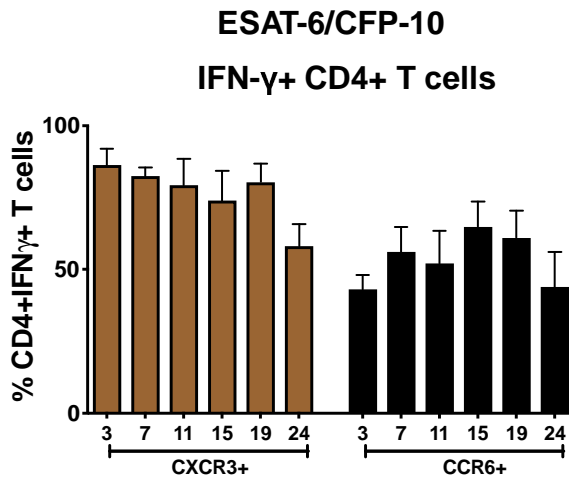




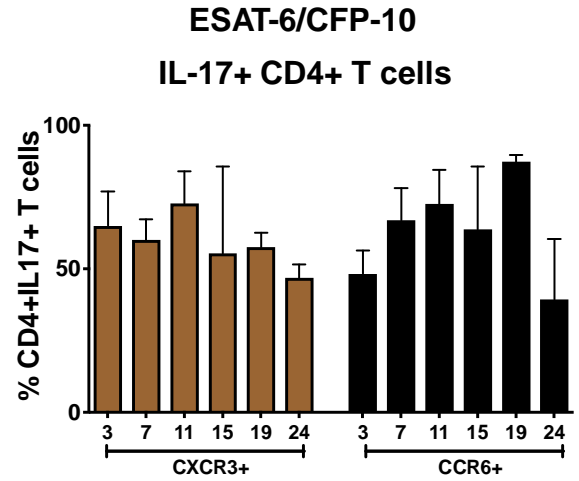


■ CXCR3+
■ CCR6+

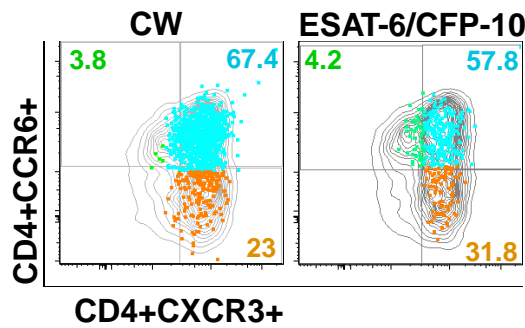
A



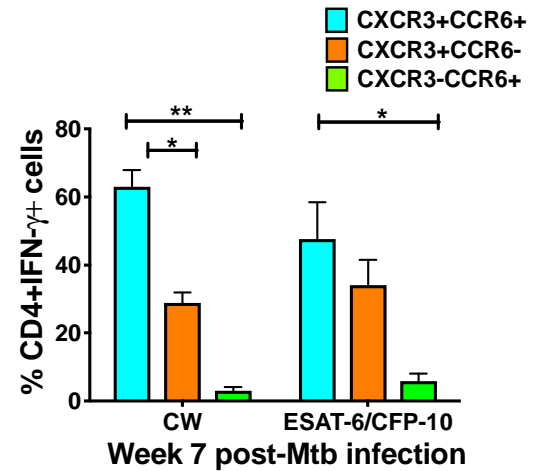
B



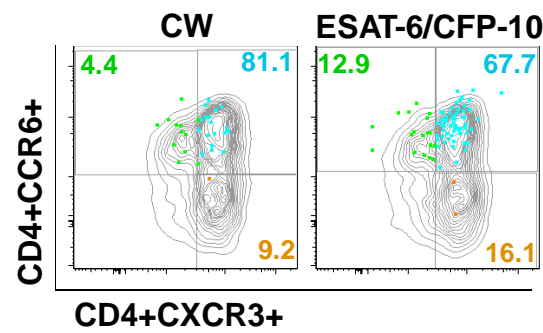
C



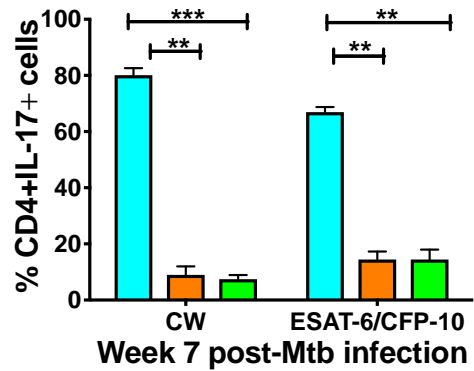
D



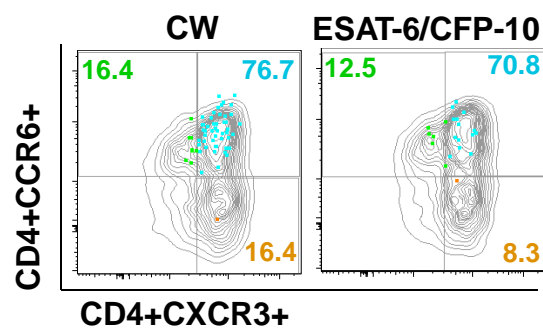
E



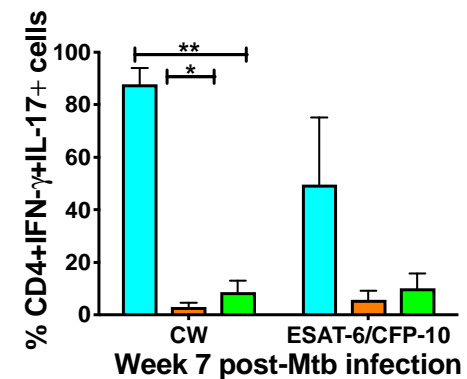
F



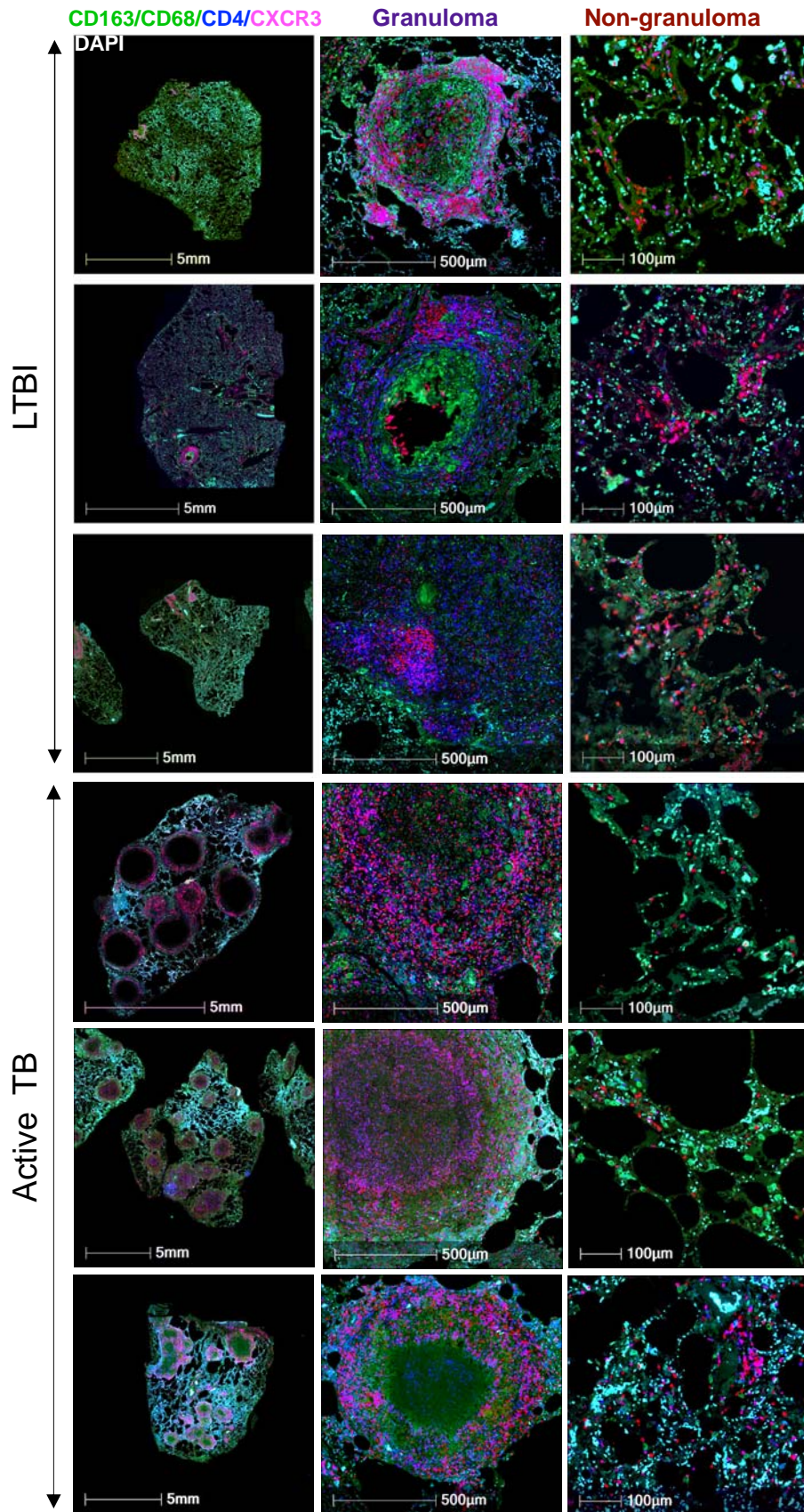
G



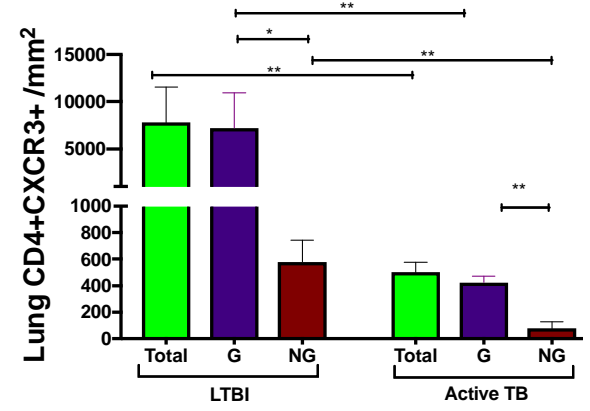
H



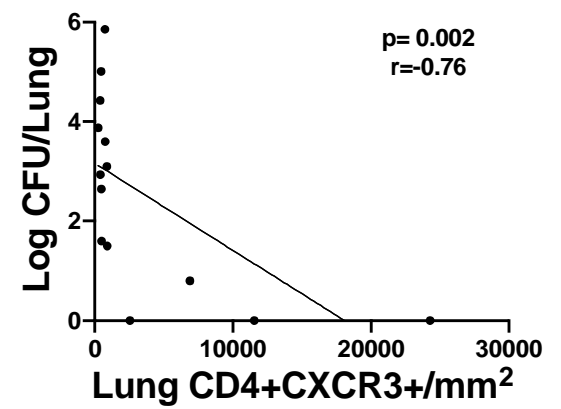
A



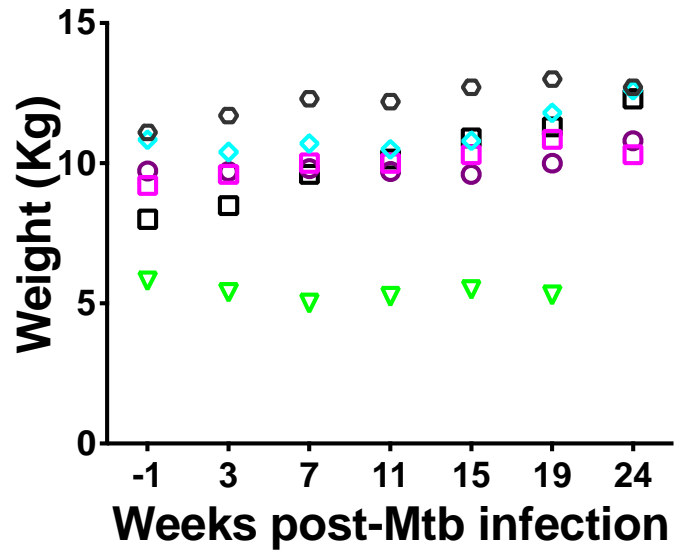
B



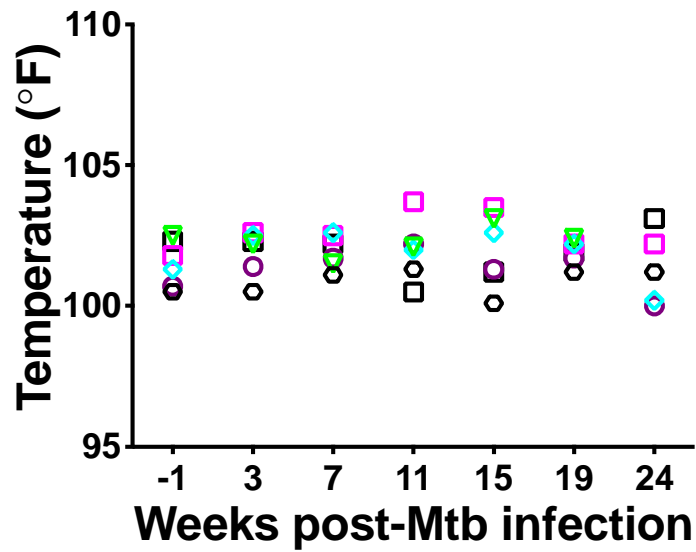
C



A

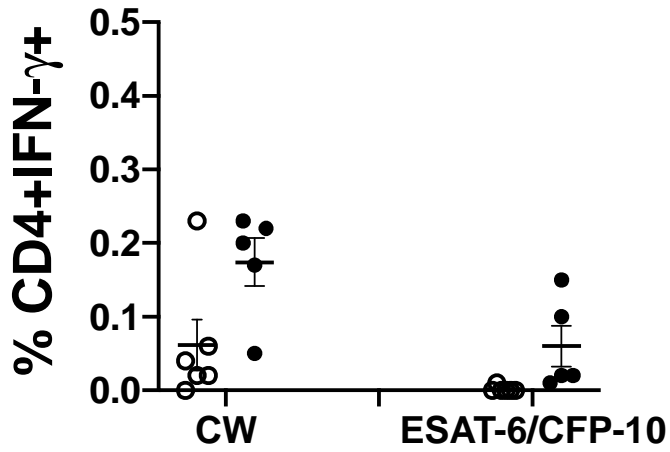


B

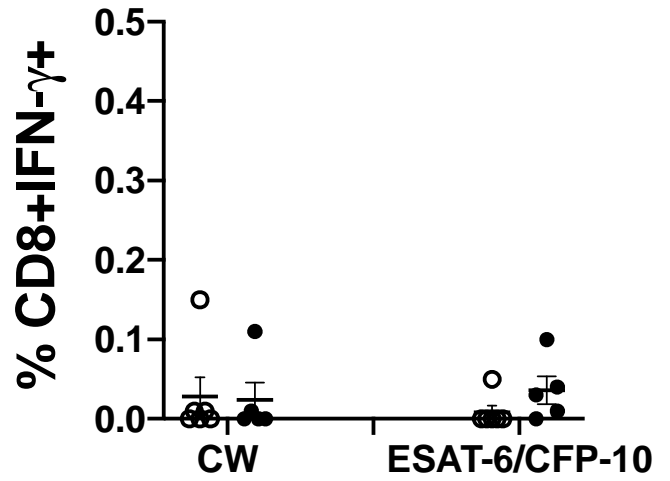


○ Week -1
● Week 3

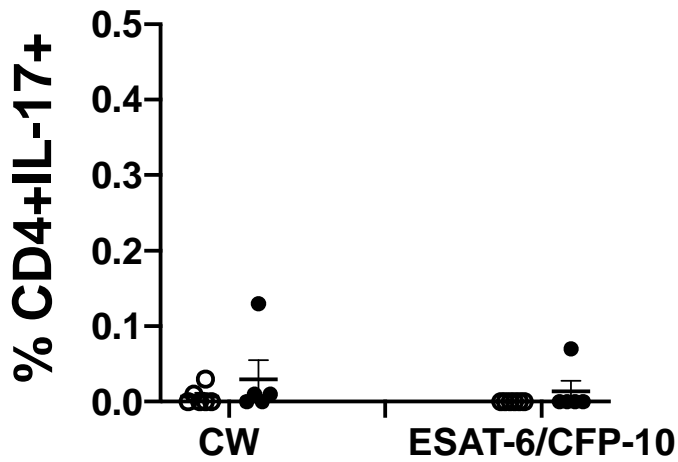
A



B



C



D

