**Title: Comparative immune responses to *Mycobacterium tuberculosis* in people with latent infection or sterilizing protection**

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30

**Abstract**

Tuberculosis (TB) affects 2 billion people worldwide and causes 1.5 million deaths every year. There is great need for new TB vaccines that are more efficacious than the currently licensed BCG vaccine, which provides only limited protection. Our goal was to identify potential targets for new TB vaccines by characterizing the immune responses that distinguish individuals with sterilizing protection against TB (TB-resisters), defined by presence of TB-specific immune responses and absence of latent infection, from individuals with latent TB infection (LTBI-participants).

Cryopreserved peripheral blood mononuclear cells (PBMC) from 13 TB-resisters and 10 LTBI-participants were analyzed by high dimensional spectral flow cytometry after overnight *M. tuberculosis (Mtb)* antigenic stimulation or unstimulated control. Activation of conventional and nonconventional T cells, NK cells, and antigen presenting cells (APC) was compared between the two groups. Compared with LTBI-participants, TB-resisters had significantly higher proportions of conventional and nonconventional T cells expressing granzyme B (GranzB) and PD-1, and of polyfunctional cells in unstimulated and *Mtb*-stimulated conditions. Conversely LTBI-participants had higher expression of CD25, CD69, CD107a, IL10 and IFNγ. An unbiased cluster analysis revealed higher frequency of recently described CD8+GMM+GranzB+ T cells in unstimulated PBMC from TB-resisters than LTBI-participants. APC activation revealed very few differences between TB-resisters and LTBI-participants. An exploratory analysis of responses in 14 BCG-recipients with minimal exposure to TB showed multiple differences with TB-resisters and LTBI-participants in PBMC activation; lower polyfunctionality of T cells and APC in *Mtb*-stimulated PBMC; and absence of CD8+GMM+GranzB+ T cells.

We conclude that combined increased T cell expression of GranzB and checkpoint inhibitors may contribute to immune protection against TB and may be targeted by new vaccines.

**Summary**

To identify immunologic targets for tuberculosis vaccines more efficacious than BCG, we investigated the immune responses that differentiate individuals with sterile protection against *M. tuberculosis (Mtb)* infection (TB-resisters) from those who develop latent tuberculosis infection (LTBI-participants). TB-resisters showed higher proportions of conventional and unconventional T cells with markers of cytotoxicity, immunologic checkpoint inhibitors, and polyfunctionality in unstimulated and ex vivo *Mtb*-stimulated mononuclear cells. Antigen presenting cells and NK cells did not significantly differentiate TB-resisters from LTBI-participants. A complementary analysis of immune responses in BCG recipients with minimal exposure to tuberculosis by virtue of having lived most of their lives in areas of low TB endemicity revealed very different unstimulated and Mtb-stimulated immunologic profiles and lower T cell and antigen presenting cell polyfunctionality compared to both TB-resisters and LTBI-participants. A combination of increased cytotoxic and regulatory responses may contribute to protection against tuberculosis and may be targeted by new vaccines.

73 **Introduction**

74 Tuberculosis (TB) is a major global health problem, since approximately 2 billion people are
75 infected with *M. tuberculosis* (*Mtb*). Vaccines are the most powerful tools for limiting the morbidity
76 and mortality of many infectious diseases, but Bacillus Calmette-Guérin (BCG)—the only licensed
77 TB vaccine—confers limited protection[1-7]. BCG administered to infants at birth is ~80% effective
78 in preventing disseminated and central nervous system TB. In contrast, BCG has variable efficacy
79 against pulmonary TB when administered to infants or adults, and many studies could not
80 demonstrate any efficacy[1-8]. BCG generates TB-specific Th1 cell-mediated immunity (CMI) that
81 has been considered essential for protection against TB, although this concept has been disputed
82 by recent studies[9-11]. For example, recent vaccine candidates that generate more robust *Mtb*-
83 specific Th1 CMI than BCG did not have greater efficacy against pulmonary TB than BCG[9, 12-
84 14]. Additional evidence that *Mtb* Th1 responses may not predict protection against *Mtb* infection
85 is that 10 to 20% of the individuals with latent TB infection, as defined by the presence of *Mtb* CMI
86 measured by tuberculin skin test (TST) or IFNγ release assays (IGRA), a measure of Th1
87 immunity, develop active TB disease over time. In contrast, 20% of individuals with household
88 contacts with highly contagious active pulmonary TB infection do not develop clinical disease and
89 have negative TST or IGRA[15]. These individuals, designated as TB-resisters, have antibodies
90 and limited CMI responses to *Mtb*, confirming prior *Mtb* infection, but their T cells do not produce
91 IFNγ in response to *Mtb* ex vivo restimulation[16, 17]. They also have genetic traits that
92 differentiate them from people with LTBI [18-21]. TB-resisters are deemed to have sterilizing
93 immunity against *Mtb*[22, 23]. However, the mechanisms of protection against *Mtb* infection in
94 TB-resisters are incompletely understood[17, 24]. Identifying these mechanisms would benefit the
95 development of new vaccines that may confer sterilizing immune protection against *Mtb*[25].

96

97 In addition to adaptive CMI, innate immunity is a critical mechanism of protection against
98 infections. Innate immunity is rapidly deployed and constitutes the first line of immune-mediated
99 defense. Moreover, innate immune responses critically contribute to the development of antigen-
100 specific adaptive CMI and establish a feedback mechanism that allows them to be also boosted
101 by adaptive CMI[26-32]. Persistent memory-like innate immune responses have been described
102 against viruses, tumors, and other antigens, including *Mtb*[28, 32-43]. Natural killer (NK) cells, γδ
103 T cells, NKT cells, invariant NKT (iNKT) cells, macrophages, monocytes (mono) and dendritic
104 cells (DC) have been shown to undergo clonal expansions and/or epigenetic modifications after
105 exposure to immunogens that can confer antigen specificity and/or allow them to activate
106 transcription programs that improve their functionality[43, 44]. Recent studies described a
107 germline encoded, mycolyl-reactive iNKT cell subset (GEMT) that recognizes the lipid glucose
108 monomycolate in the context of CD1b and participates in the elimination of *Mtb* from the host[40,
109 42, 45]. Compared to other iNKT, CD1b-restricted GEMT cells have a more diverse T-cell receptor
110 repertoire, which permitted the identification of GEMT cell clonal expansions in patients
111 recovering from TB and their persistence in the host for several years after *Mtb* elimination[40,
112 42]. Another nonconventional T cell subset of interest is the mucosal-associated invariant T cells
113 (MAIT) that developmentally share some characteristics both with iNKT and γδ T cells. MAIT can
114 recognize microbial riboflavin derivatives presented by the major histocompatibility-related
115 receptor 1 (MR1) and play a critical role in antibacterial, including *Mtb*, pulmonary defenses via
116 cytokine production and cytotoxicity[46-48]. MR1+ MAIT were shown to account for most IFNγ
117 production in response to BCG[49]. MR1- MAIT lack the receptor for antigen recognition and are
118 deemed to respond to secreted cytokines[50, 51]. The role of innate immune responses in
119 sterilizing immunity against *Mtb* infection has not been studied.

120

121 The overarching goal of our study was to identify innate and adaptive immune responses that
122 differentiate TB-resisters from people with LTBI (LTBI-participants) among household contacts of
123 active pulmonary TB cases. We leveraged a longitudinal cohort study of household contacts of
124 individuals with active pulmonary TB disease [Cohort For TB Research By The Indo-US Medical
125 Partnership Multicentric Prospective Observational Study (C-TRIUMPH)] in Pune, India, by using
126 advanced spectral flow cytometry technology and high-dimensional analytic tools on peripheral
127 blood mononuclear cells (PBMC) archived from the study participants[23, 52, 53]. To further
128 understand how the responses to *Mtb* in LTBI-participants and TB-resisters may differ from those
129 induced by BCG, we also included in our analysis a group with documented BCG vaccination and
130 with minimal exposure to TB.

131

132 **Results**

133 *Characteristics of the study population*

134 The study used PBMC from 13 TB-resisters and 11 LTBI-participants in C-TRIUMPH, who had
135 intense exposure to active pulmonary TB household contacts (**Table 1**). Importantly, TB-resisters
136 and LTBI-participants had similarly high exposure scores to TB (median=7), a measure of the
137 likelihood of acquiring *Mtb* infection from a household index case[54]. Also recruited were 14
138 BCG-recipients with limited exposure to TB by virtue of having spent most of their lives in the
139 United States or other countries with low incidence of TB. In addition to these differences in
140 upbringing and environmental exposures, we noted that BCG-recipients significantly differed from
141 C-TRIUMPH participants in race/ethnicity, but not in age or BMI.

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146 **Table 1. Characteristics of the study population**

147

| | TB-Resisters | LTBI+ participants | BCG | P-Value* (TB-Resisters vs. LTBI+) | P-Value* (three-group) |
|---|---|---|---|---|---|
| N | 13 | 11 | 14 | | |
| Median years of age (range) | 24 (9, 65) | 29 (7, 62) | 29 (20, 41) | 0.81 | 0.71 |
| N Female sex (%) | 6 (46) | 4 (36) | 10 (71.4) | 0.70 | 0.19 |
| N Race and Ethnicity (%) | | | | | |
| Asian (Indian subcontinent) | 13 (100) | 11 (100) | 0 (0) | | |
| Asian (not Indian subcontinent) | 0 (0) | 0 (0) | 3 (21.4) | na | <0.0001 |
| Black | 0 (0) | 0 (0) | 2 (14.3) | | |
| Hispanic Non-Black | 0 (0) | 0 (0) | 5 (35.7) | | |
| Non-Hispanic White | 0 (0) | 0 (0) | 4 (28.6) | | |
| Median BMI (range) | 23.3 (13.1, 30.5) | 20.4 (13.5, 29.8) | 22.8 (20, 30.6) | 0.49 | 0.49 |
| Median exposure score (range) | 7 (4, 9) | 7 (4, 9) | na | 0.84 | na |
| N with BCG scar (%) | 9 (64.3) | 5 (35.7) | na | 0.24 | na |

148

149 ***** ANOVA for continuous variables and Fisher's exact for categorical variables.

150 **Abbreviations**: BCG=Bacille de Calmette-Guerin; BMI=Body mass index (kg/m$^2$); LTBI=latent
151 tuberculosis infection; N=number; na=not applicable

152

153

154 *Phenotypic and functional characteristics of innate and adaptive immune responses in TB-*
155 *resisters and LTBI-participants*

156 We used two high dimensional flow cytometry panels [T-cell and antigen-presenting-cell (APC)]
157 on PBMC stimulated with *Mtb* antigen or unstimulated controls (**Table S1**). In the T-cell panel, we
158 characterized CD4+ and CD8+ conventional T cells (Tconv), NKT, γδ T, GEMT, iNKT, MR1+ and
159 MR1- MAIT, and NK cells. Functionality was assessed by expression of CD25 and CD69
160 activation markers; PD1 immunologic checkpoint receptor; CD107a (lysosome degranulation)
161 and granzyme B (GranzB) cytotoxicity markers; GMCSF, IL2, IL10, IL17, IFNγ, and TNFα
162 cytokines; and Ki67 proliferation marker. In the APC panel, we characterized functionality of
163 CD14+ monocytes (Mono), CD123+ plasmacytoid DC (pDC), CD11c+CD14- total conventional

164 DC (cDC total), CD141+ cDC1 and CD1c+ cDC2 subsets using the following functional readouts:
165 CD40, CD80 and CD83 activation markers; PDL1 immunologic checkpoint ligand; and GMCSF,
166 IL1β, IL8, IL10, IL12p40, IL27 and TNFα cytokines. Gating trees are shown in **Fig S1**.
167
168 In *unstimulated* conditions, 10 functional cell subsets had significantly higher frequencies in TB-
169 resisters compared with LTBI-participants, consisting of GranzB-expressing CD4+ Tconv, CD8+
170 Tconv, CD8+ iNKT and CD8+ NKT; PD1-expressing CD4+ Tconv, CD8+ Tconv, CD8+ iNKT and
171 MR1- MAIT; CD40+ pDC; and IL1β+ Monos (**Fig 1**). In contrast, we only detected a single subset
172 (Ki67+MR1+ MAIT) with significantly higher frequencies in LTBI-participants compared with TB-
173 resisters.
174
175 Many of the functional differences between TB-resisters and LTBI-participants observed in
176 unstimulated PBMC persisted after *Mtb* ex vivo stimulation (**Fig 2**). Differences between TB-
177 resisters and LTBI-participants in *Mtb-stimulated* PBMC included higher proportions of (1)
178 GranzB-expressing CD4+ Tconv, CD8+ Tconv, CD8+ iNKT. CD8+ NKT and MR1- MAIT cells;
179 and (2) PD1-expressing CD4+ Tconv, CD8+ Tconv and MR1- MAIT in TB-resisters; and higher
180 proportions of (1) CD25-expressing CD4+ and CD8+ Tconv and NKT, CD8+ iNKT, and MAIT
181 MR1-; (2) CD107+CD8+ iNKT and NKT; (3) IFNγ-expressing CD4+ and CD8+ iNKT; (4)
182 CD8+IL10+ iNKT; and (5) CD69+ γδ T cells in LTBI-participants.
183
184 *Mtb-memory* responses were characterized by subtracting frequencies of unstimulated from *Mtb-*
185 *stimulated* corresponding PBMC. Using this definition, we identified 6 differentially expressed
186 functional subsets with significantly higher frequencies in LTBI-participants compared with TB-
187 resisters: CD25+ and CD69+ γδ T cells; CD4+CD25+ Tconv; CD8+IFNγ+ iNKT; and

188 CD8+CD107a+ NKT (**Fig 3**). There were no *Mtb-memory* subsets with higher frequency in TB-
189 resisters than in LTBI-participants.
190
191 *Polyfunctionality of the immune cell subsets in TB-resisters and LTBI-participants*
192 Polyfunctionality analyses based on Boolean gating of CD25, CD107a, GranzB, IFNγ, IL10 and
193 PD1 in the T cell panel showed higher marker co-expression in TB-resisters than in LTBI-
194 participants in *unstimulated* conditions on CD8+ iNKT, MR1- MAIT, CD8+ NKT and CD4+ and
195 CD8+ Tconv (**Fig 4A**). In *Mtb-stimulated* conditions, CD8+ iNKT, MR1- MAIT, CD8+ NKT, and
196 CD4+ and CD8+ Tconv maintained higher polyfunctionality in TB-resisters than LTBI-participants.
197 LTBI-participants did not have any subsets with higher polyfunctionality than TB-resisters. There
198 were no differences in polyfunctionality of NK and γδ T cells in Mtb-stimulated or unstimulated
199 conditions between TB-resisters and LTBI-participants (**Fig S2**).
200
201 In the APC analysis, we evaluated the expression of CD40, CD80, IL1β, IL27, PDL1 and TNFα
202 on Mono, cDC1, cDC2, cDC total, and pDC. **Figure 4B** shows that TB-resisters had higher
203 proportions of polyfunctional Mono than LTBI-participants in *unstimulated* conditions. No other
204 differences were observed between TB-resisters and LTBI-participants.
205
206 *Coordination of the immune responses in TB-resisters and LTBI-participants*
207
208 We complemented the analysis of manually gated PBMC subsets with Phenograph unbiased
209 analysis, which identified 8 T-cell and 1 APC clusters that significantly differed between TB-
210 resisters and LTBI-participants in *unstimulated* PBMC; 3 T-cell and 1 APC clusters in *Mtb-*
211 *stimulated* conditions; and 5 T-cell and 3 APC *Mtb-memory* clusters (**Fig S3**). Most clusters
212 confirmed findings already observed in the manually gated analysis, with the following exceptions:
213 (1) Two T-cell and one APC *Mtb-memory* clusters (**Fig S3**) with higher relative frequencies in TB-

214 resisters than LTBI-participants, in contrast to the manual gating analysis that did not identify
215 excess *Mtb-memory* subsets in TB-resisters compared with LTBI-participants; and (2) A
216 CD8+GMM+GranzB+ Tconv subset that had not been included in the prespecified manual gating
217 analysis (**Fig 5 and S3**). This subset had higher frequency in *unstimulated* PBMC from TB-
218 resisters compared with LTBI-participants (FDR-corrected p=0.008).
219

220 *Immune responses in BCG-recipients*

221

222 The analysis of functional phenotypes in BCG-recipients showed significant differences with TB-
223 resisters in 54 *unstimulated,* 41 *Mtb-stimulated*, and 41 *Mtb-memory* PBMC subset responses
224 (**Fig S4**). Compared to LTBI-participants, BCG-recipients showed differences in 56 *unstimulated*,
225 32 *Mtb-stimulated*, and 24 *Mtb-memory* responses (**Fig S5**).
226

227 The comparison of polyfunctional NK and T cells in BCG-recipients and TB-resisters showed
228 significantly higher frequencies of polyfunctional MR1+ and MR1- MAIT and CD4+ and CD8+
229 NKT and Tconv in TB-resisters both in *unstimulated* and *Mtb-stimulated* PBMC, while BCG-
230 recipients had higher frequencies of polyfunctional CD4+ iNKT cells (**Fig 6A**). Compared with
231 BCG-recipients, LTBI-participants had higher frequencies of polyfunctional MR1+ MAIT cells in
232 *unstimulated* PBMC, while BCG-recipients had higher frequencies of CD4+ iNKT (**Fig 6B**). In *Mtb-*
233 *stimulated* conditions, LTBI-participants showed higher frequencies of polyfunctional MR1+ MAIT
234 and CD4+ Tconv, while BCG-recipients had higher frequencies of polyfunctional CD4+ and CD8+
235 iNKT.
236

237 The polyfunctionality analysis of APC showed that compared with BCG-recipients, TB-resisters
238 had higher proportions of polyfunctional Monos, cDC1, cDC2 and pDC in *unstimulated* PBMC

239 (**Fig 6C**). After *Mtb-stimulation* the cDC1 and cDC2 also had higher polyfunctionality in TB-
240 resisters and none of the APC subsets had higher polyfunctionality in BCG-recipients. Compared
241 to BCG-recipients LTBI-participants had higher frequencies of polyfunctional cDC1 in
242 *unstimulated* PBMC, while BCG-recipients had higher frequencies of polyfunctional cDC1 and
243 cDC2 after *Mtb stimulation* (**Fig 6D**).
244
245 One of the two recent descriptions of GMM+ Tconv showed their presence in response to BCG
246 administration in infants, but not in adults[55]. On comparing the frequency of
247 CD8+GMM+GranzB+ T cells in BCG-recipients with TB-resisters and LTBI-participants in our
248 study, we also found very small frequencies of this cell subset (≤0.005% out of total lymphocytes)
249 in BCG-recipients, significantly lower compared with either of the *Mtb*-exposed groups (FDR-
250 corrected p≤0.00013; **Fig S7**).
251
252
253 **Discussion**
254
255 The goal of this study was to characterize responses unique to TB-resisters compared to LTBI-
256 participants that may be targeted by new TB vaccines. A consistent finding was increased
257 GranzB-expressing conventional and nonconventional T cells in *Mtb-stimulated* and *unstimulated*
258 PBMC of TB-resisters. This hallmark was observed in the prespecified manual gating and in the
259 unsupervised cluster analyses and included expression of GranzB by Tconv, NKT and iNKT cells.
260 The *Mtb-memory* analysis reveal higher cytotoxic responses in TB-resisters using the unbiased
261 cluster analysis but not the manual gating. Collectively, these findings suggest that TB-resisters
262 may mount higher and/or faster cytotoxic responses upon exposure to *Mtb* than LTBI-participants,
263 which may contribute to the clearance of the infectious agent before establishing latency.
264

265 Of special interest was a population of CD8+GMM+GranzB+ Tconv that was not part of the
266 prespecified manual gating but was identified by the Phenograph cluster analysis. This cell
267 subset, which has only recently been described, was found in people with active TB, in whom
268 CD8+GMM+ T cells displayed upregulated cytotoxic transcription profiles[56]. The authors
269 concluded that this cell subset may contribute to the clearance of *Mtb*-infected host cells. Notably,
270 we found higher proportions of CD8+GMM+GranzB+ T cells in TB-resisters compared with LTBI-
271 participants, suggesting that this cell subset may contribute to the sterilizing immune protection
272 of TB-resisters against *Mtb* infection.
273
274 TB-resisters also had higher proportions of PD1-expressing Tconv, iNKT and MR1- MAIT than
275 LTBI-participants in *unstimulated* PBMC and after ex vivo *Mtb stimulation*. PD1 is an immunologic
276 checkpoint inhibitor commonly expressed on activated T cells[57]. PD1 expression contributes to
277 quenching the immune response after removal of the stimulating agent, limiting the inflammatory
278 response and tissue destruction. Recent reports showed an association of increased T cell
279 activation with TB morbidity and with increased risk of active *Mtb* infection in BCG-recipients[58-
280 60]. Moreover, PD1 deficient mice as well as people treated with PD1/PDL1 blocking agents have
281 increased risk of *Mtb* infection, morbidity, and reactivation[61-63]. Collectively, these observations
282 suggest that the increased expression of PD1 in TB-resisters may limit their susceptibility to *Mtb*
283 tissue destruction and, perhaps, propagation of infection.
284
285 A distinguishing characteristic of the immune response in TB-resisters from LTBI-participants was
286 polyfunctionality. TB-resisters had higher proportions of polyfunctional Tconv and
287 nonconventional T cells both in *Mtb-stimulated* and *unstimulated* conditions. Polyfunctional CD4+
288 Tconv, characterized by expression of IL2, IFN$\gamma$ and/or TNF$\alpha$ have been previously associated
289 with protection against active TB infection in animal models[64]. However, the role of
290 polyfunctional CD4+ T cell responses in protection against *Mtb* infection in humans remains

291 uncertain, particularly since a recent study showed that IL2, IFNγ and/or TNFα polyfunctional
292 CD4+ T cell responses generated by BCG did not correlate with protection against TB disease in
293 vaccinated infants[11]. In our study, cytokine production made a minor contribution to the
294 polyfunctional T cell responses, which predominantly expressed GranzB, CD107a, CD25 and
295 PD1, and involved mostly CD8+ T cells, including NKT, iNKT and Tconv. In addition, CD4+ Tconv
296 and MR1- MAIT cells also showed higher polyfunctionality in TB-resisters than LTBI-participants
297 expressing the same markers as the CD8+ Tconv. These findings suggest that high proportions
298 of cytotoxic Tconv and nonconventional T cells may contribute to the immune protection of TB-
299 resisters against *Mtb* infection.
300
301 *Mtb-memory* T cell responses identified by the expression of single activation markers were
302 higher in LTBI-participants than in TB-resisters, although two *Mtb-memory* CD4+ Tconv clusters
303 revealed by the Phenograph analysis had higher frequencies in TB-resisters than in LTBI-
304 participants. These data confirm that TB-resisters develop *Mtb*-specific T cell memory, but in
305 general *Mtb-memory* responses are higher in LTBI-participants. This finding may be related to
306 the observation that 25% of people with LTBI have evidence of active *Mtb* replication, which
307 continuously exposes the immune system to *Mtb* antigens and maintains high levels of memory
308 cells[65-68].
309
310 APC displayed few differentiating features between TB-resisters and LTBI-participants. In
311 *unstimulated* PBMC, TB-resisters had higher frequencies of activated and polyfunctional Mono
312 by manual gating and Boolean analyses, respectively, and cDC2 by cluster analysis. However,
313 no differences were found in *Mtb-stimulated* responses between TB-resisters and LTBI-
314 participants. It is important to note that *Mtb* contains multiple toll-like receptor (TLR) agonists,
315 which hinders the discrimination of *Mtb-stimulated* responses from nonspecific stimulation TLR-

316 mediated activation of APC. Nevertheless, the Phenograph analysis identified an activated Mono
317 cluster with higher frequency in TB-resisters than LTBI-participants.
318
319 The analysis of *Mtb-memory* responses in BCG-recipients using single markers of activation
320 showed both higher and lower responses in multiple T-cell and APC subsets compared with TB-
321 resisters or LTBI-participants. These differences may have been related to the difference in
322 environmental exposures, including *Mtb*, in addition to genetic backgrounds of BCG-recipients
323 compared with TB-resisters or LTBI-participants. The Boolean analysis of polyfunctional
324 responses narrowed down the differences between BCG-recipients and LTBI-participants or TB-
325 resisters. Overall, BCG-recipients had higher frequencies of *unstimulated* and *Mtb-stimulated*
326 iNKT polyfunctional cells than both LTBI-participants and TB-resisters. BCG-recipients also had
327 lower frequencies of *unstimulated* and/or *Mtb-stimulated* Tconv, $\gamma\delta$, NKT, and/or MAIT
328 polyfunctional cells compared to both LTBI-participants and TB-resisters, but a larger number of
329 polyfunctional subsets differentiated BCG-recipients from TB-resisters than LTBI-participants.
330 Compared to TB-resisters, BCG-recipients also had universally lower frequencies of *stimulated*
331 and *Mtb-stimulated* polyfunctional APC subsets. Compared to LTBI-participants, BCG-recipients
332 had lower frequencies only of *stimulated* and *Mtb-stimulated* polyfunctional cDC1 and higher
333 frequencies of polyfunctional cDC2. Collectively, these findings suggest superior polyfunctionality
334 of T cells and APC in TB-resisters compared with BCG-recipients.
335
336 Our study has both strengths and limitations. The main limitations consisted of the small number
337 of participants and different demographic features of BCG-recipients compared with C-TRIUMPH
338 participants (e.g., race/ethnicity). Although our study did not measure antibody responses, which
339 have been recently shown to differentiate TB-resisters from LTBI-participants[17, 69], the CMI
340 analysis was comprehensive and revealed new findings unique in TB-resisters.
341

342 We conclude that both innate and adaptive responses distinguish TB-resisters from LTBI-
343 participants. Unique elements of the cell-mediated immune responses to *Mtb* in TB-resisters are
344 high cytotoxic potential, expression of immunologic checkpoint inhibitors and polyfunctionality of
345 Tconv and nonconventional T cells, suggesting that some or all may be key factors in an effective
346 immune response against *Mtb*. Although TB-resisters displayed these characteristics even
347 without ex vivo *Mtb stimulation*, it is reasonable to propose that a vaccine that increases some or
348 all these responses in the form of *Mtb-memory* may be more effective than BCG.
349

350 **Materials and Methods**

351 Participants. Cryopreserved PBMC were obtained from 13 TB-resisters and 11 LTBI-participants
352 in C-TRIUMPH [23, 52, 53]. LTBI-participants were defined by positive IGRA and/or TST at entry.
353 TB-resisters had negative IGRA and TST at entry and over the 2 years of follow up. BCG-
354 recipients whose immunization status was documented by medical history, who lived most of their
355 lives outside of TB endemic areas, and who had negative IGRA were enrolled at the University of
356 Colorado Anschutz Medical Campus (CU-AMC).
357 IGRA testing used Quantiferon Gold in Tube kit (Qiagen) performed as per manufacturer's
358 instructions.
359 Flow Cytometry. Testing conditions were optimized using a convenience sample of PBMC from
360 LTBI-participants and donors without previous exposure to TB or BCG submitted to stimulation
361 for several time intervals with different concentrations of irradiated *Mtb*, whole cell lysate, cell
362 membrane, PPD and BCG. *Mtb* cell membrane provided the best discrimination between LTBI-
363 participants and TB-unexposed and was selected for this study. PBMC were thawed in serum-
364 free media (AIM V, Gibco) supplemented with benzonase (50 units/mL, Millipore). PBMC were
365 resuspended at $2*10^6$ cells/ml and stimulated with cell membrane (BEI Resources Strain
366 CDC1551, Cell Membrane Fraction, NR-14832) at 20ug/ml in AIM V or unstimulated overnight.

367 Brefeldin-A and Monensin (SIGMA) at 5ug/ml each were added for the last 4h of culture. For the
368 T-cell panel, CD107a-BV785 (Biolegend) was added for the last 4h of culture. Cells were washed
369 with PBS, stained with Zombie NIR Fixable Viability dye (Biolegend), and washed with PBS+1%
370 albumin. For the APC panel, cells were treated with Human TruStain FcX (Biolegend), surface-
371 stained with CD16-eFluor450, CD83-PerCP-eFluor710 (Thermo Fisher/eBioscience), CD40-
372 BV480, CD141-BV750, CD11c-BB515 (BD Biosciences), CD80-BV510, CD1c-BV605, CD123-
373 BV650, PDL1-BV785, HLA-DR-AlexaFluor488, CD3-, CD19-, CD20- and CD56-PerCP-Cy5.5,
374 CD14-APC-Fire750 (Biolegend), brilliant stain buffer plus (BD Biosciences) and True-stain
375 monocyte blocker (Biolegend). Cells were washed and treated with eBioscience Foxp3 Staining
376 Buffer Set (Thermo Fisher/eBioscience), then stained with IL8-BV421, IL10-BV711, IL1β-PE (BD
377 Biosciences), GMCSF-PE-Dazzle594, IL12p40-AlexaFluor647 (Biolegend), IL27-APC, TNFα-
378 AlexaFluor700 (Thermo Fisher/eBioscience) and brilliant stain buffer plus. For the T-cell panel,
379 cells were treated with 50% human AB serum (Gemini) in PBS+1% albumin, washed and surface-
380 stained with tetramers: CD1b-unloaded-FITC, CD1b-GMM-APC, and MR1-BV421. Cells were
381 washed, stained with TCR Vα7.2-BV605, TCR Vα24-Jα18-PE-Cy7 (Biolegend) and TCR γδ-PE
382 (BD Biosciences) followed by CD16-eFluor450, CD3-AlexaFluor532 (Thermo
383 Fisher/eBioscience), CD56-BV480, CD25-BB515 (BD Biosciences), CD8-BV510, PD1-BV711,
384 CD69-PE-Cy5, CD161-AlexaFluor700 (Biolegend) and brilliant stain buffer plus. Cells were
385 washed, treated with Foxp3 Staining Buffer Set, then stained with IL17-BV570, IL2-BV650, IL10-
386 PE-Dazzle594, GranzymeB-APC-Fire750 (Biolegend), IFNγ-BV750, GMCSF-AlexaFluor647 (BD
387 Biosciences), Ki67-PerCP-eFluor710, TNFα-PerCP-Cy5.5 (Thermo Fisher/eBioscience) and
388 brilliant stain buffer plus. Cells from both panels were washed and fixed with PBS+1%
389 paraformaldehyde, acquired on the Cytek Aurora™ (Cytek Biosciences), and analyzed with
390 FlowJo (Becton Dickinson). Two leucopack controls were used in each run to ensure inter-run
391 reproducibility and to examine technical effects.

392 Boolean analysis. Boolean combinatorial gates were created using the 6 cytokines/activation
393 markers, generating 64 distinct activation phenotypes. Graphical representation was performed
394 using SPICE 6.0 software[70]. The data were analyzed using permutations (10,000 iterations)
395 included in the software.
396
397 UMAP analysis. For T cell clustering, we used 67,000 down-sampled events from 13 TB-resisters
398 and 9 LTBI-participants for a total of 2,948,000 events in the concatenated file. For APC
399 clustering, we used data from 11 TB-resisters and 8 LTBI-participants for a total of 502,479 events
400 in the concatenated file. Parameters were rescaled to ArcSinh prior to running UMAP with
401 phenograph in FlowJo on the concatenated file using parameters, Euclidean distance, 15 nearest
402 neighbors and minimum distance of 0.5[71].
403 Statistical Testing of Cell Lineage and Function Between TB-Resisters and LTBI-participants**.** To
404 compare the average cell lineage frequencies obtained from manual and data-driven gating
405 between groups, we used beta regression (*betareg* R package, v3.1-3) with a logit-link to model
406 cell lineage percent (regression outcome; between 0 and 100% of events) on group (primary
407 explanatory variable of interest; LTBI-participant versus TB-resisters), adjusting for participant
408 age in years as a covariate [72]. Similarly, to test for functional differences among *unstimulated*
409 conditions, we modeled percent positivity (outcome; between 0 and 100% of cells in given lineage
410 expressing functional marker) on group, correcting for age. “Rare” outcomes (i.e., outcomes of
411 0% in over 1/3$^{rd}$ of participants) were excluded from testing due to their low variability among our
412 participants. For each set of statistical tests, we defined significant between-group differences as
413 a non-zero group regression coefficient (Wald test, multiple testing adjustment using the
414 Benjamini-Hochberg false discovery rate < 0.05)[73].
415 To examine differences in functional markers under *Mtb-stimulated* conditions, we
416 repeated this modeling procedure but with the stimulated percent positivity values as the
417 regression outcome variable, adjusting for age. We also examined differences in *Mtb-memory* by

418 additionally adjusting for each participant's corresponding baseline values (unstimulated percent
419 positivity values in their paired control sample) as a covariate. The *Mtb-memory* analysis thus
420 emphasizes markers that were not already differentially abundant between groups prior to
421 stimulation, or stimulation effects potentially masked by unstimulated differences. Adjusting for
422 baseline levels are conceptually similar to "subtracting" the unstimulated values from the Mtb-
423 stimulated values; as a consequence, we visualize Mtb-memory with Δ-values, defined by
424 stimulated frequencies minus unstimulated frequencies. Functional markers with limited changes
425 following Mtb-stimulation (i.e., Δ-values of magnitude ≤ 0.1% in over 1/3$^{rd}$ of participants) were
426 excluded from testing due to their low variability among our participants and tendency to
427 recapitulate unstimulated tests. Significant between-group differences in Mtb-memory were
428 based on FDR < 0.05.

429 These modeling procedures was also repeated for the TB-resister versus BCG recipient
430 comparisons and the LTBI-participants versus BCG recipient comparisons. All analyses were
431 performed in the R statistical package v4.0.2 unless otherwise specified. Additional
432 methodological details and analysis code are publicly available at
433 https://github.com/chooliu/Sterile_vs_Latent_TB_Immunity.

434

435 **Declaration of approval**. The study was approved by the Colorado Multiple Institutional Review
436 Board (COMIRB) and by the IRBs of the Byramjee Jeejeebhoy Govt. Medical College, India and
437 Johns Hopkins Medicine. All participants signed informed consent.

438

439 **Author contributions**: AW designed the study, oversaw the laboratory assays, analyzed the
440 data and prepared the manuscript. EJ developed the flow cytometry assays, analyzed the data
441 and contributed to manuscript preparation. CL performed the statistical analysis and contributed
442 to manuscript preparation. NL conducted regulatory and clinical activities at CU-AMC. For the
443 clinical research cohort activities in Pune, India: VM, MP, RL, CV oversaw the clinical enrollment

444 and follow-up activities; VK, RB, AK conducted the laboratory activities and specimen
445 management; NG conducted the data management activities; MP oversaw all the day-to-day
446 study coordination activities; and AG, VM obtained the funding for the clinical cohort protocol,
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474 https://main.ccghe.net/content/report-c-triumph-cohort-tuberculosis-research-indo-us-medical-
475 partnership.
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759

760 **Supporting Information**

761

762 **Table S1. Flow cytometry panels**

763

764 **Figure S1. Gating strategy**. **Top**: T cells including NK cells. **Bottom**: APC.

765

766 **Figure S2. Polyfunctionality of the immune responses to Mtb in TB-resisters (LTBIneg)**
767 **compared with LTBI-participants (LTBIpos).** Data were derived from 13 TB-resisters and 11
768 LTBI+ participants. Pie charts 1-4 show proportions of cells expressing no markers (dark blue) or
769 ≥1 marker (other colors) in response to Mtb stimulation or control. Arches show the markers
770 expressed by the responding cells. The permutation tables present p values for comparisons
771 across TB-resisters unstimulated (1), TB-resisters Mtb-stimulated (2), LTBI+ participants
772 unstimulated (3) and LTBI+ participants Mtb-stimulated (4) conditions. Tables show unadjusted
773 p-values for each comparison. Pink boxes indicate significant differences defined by FDR $p <$
774 $0.05$.

775

776 **Fig S3. Cluster analysis of adaptive and innate immune responses in TB-resisters and**
777 **LTBI-participants.** Data were derived from 13 TB-resisters and 9 LTBI-participants for the T cell
778 clustering and 12 TB-resisters and 8 LTBI-participants for the APC clustering. **A**: Forest trees of
779 lnOR and 95% CI, means, and FDR-corrected p values of clusters significantly different between
780 the two groups. **B**: Heat map of T-cell clusters identified by Phenograph. **C**: Heat map of APC
781 clusters.

782

783 **Figure S4**. **Comparison of immune cell subset frequencies in BCG recipients and TB-**
784 **Resisters.** Data were derived from 13 TB-Resisters and 14 BCG recipients. Forest plot displays
785 ln-transformed odds ratios (lnOR) and 95% confidence intervals (CI). The dotted line shows no

786 average effect (lnOR = 0, corresponding to OR = 1). Features on the right side of this line are
787 more highly expressed among TB-Resisters (OR > 1), and features on the left side are more
788 highly expressed among BCG recipients (OR < 1). The table shows the absolute OR and the
789 means of each parameter in TB-resisters and LTBI-participants. **Top**: unstimulated PBMC;
790 **Middle**: Mtb-stimulated; **Bottom**: Mtb-memory
791
792 **Figure S5**. **Comparison of immune cell subset frequencies in BCG recipients and LTBI-**
793 **articipants.** Data were derived from 11 LTBI-participants and 14 BCG-recipients. Forest plot
794 displays ln-transformed odds ratios (lnOR) and 95% confidence intervals (CI). The dotted line
795 shows no average effect (lnOR = 0, corresponding to OR = 1). Features on the right side of this
796 line are more highly expressed among LTBI-participants (OR > 1), and features on the left side
797 are more highly expressed among BCG recipients (OR < 1). The table shows the absolute OR
798 and the means of each parameter in TB-resisters and LTBI-participants. **Top**: unstimulated
799 PBMC; **Middle**: Mtb-stimulated; **Bottom**: Mtb-memory.
800
801 **Figure S6. Polyfunctionality of select T cell responses in BCG-recipients by comparison**
802 **with TB-resisters (LTBIneg) and LTBI-participants (LTBIpos).** Data were derived from 14
803 BCG-recipients, 13 TB-resisters, and 11 LTBI-participants. **A:** BCG-recipients and TB-resisters;
804 **B**: BCG-recipients and LTBI-participants. Pie charts 1-4 show proportions of cells expressing no
805 markers (dark blue) or ≥1 marker (other colors) in response to Mtb stimulation or control. Arches
806 show the markers expressed by the responding cells. The permutation tables present p values
807 for comparisons across all groups and conditions. Tables show unadjusted p-values for each
808 comparison. Pink boxes indicate significant differences defined by FDR $p < 0.05$.
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810
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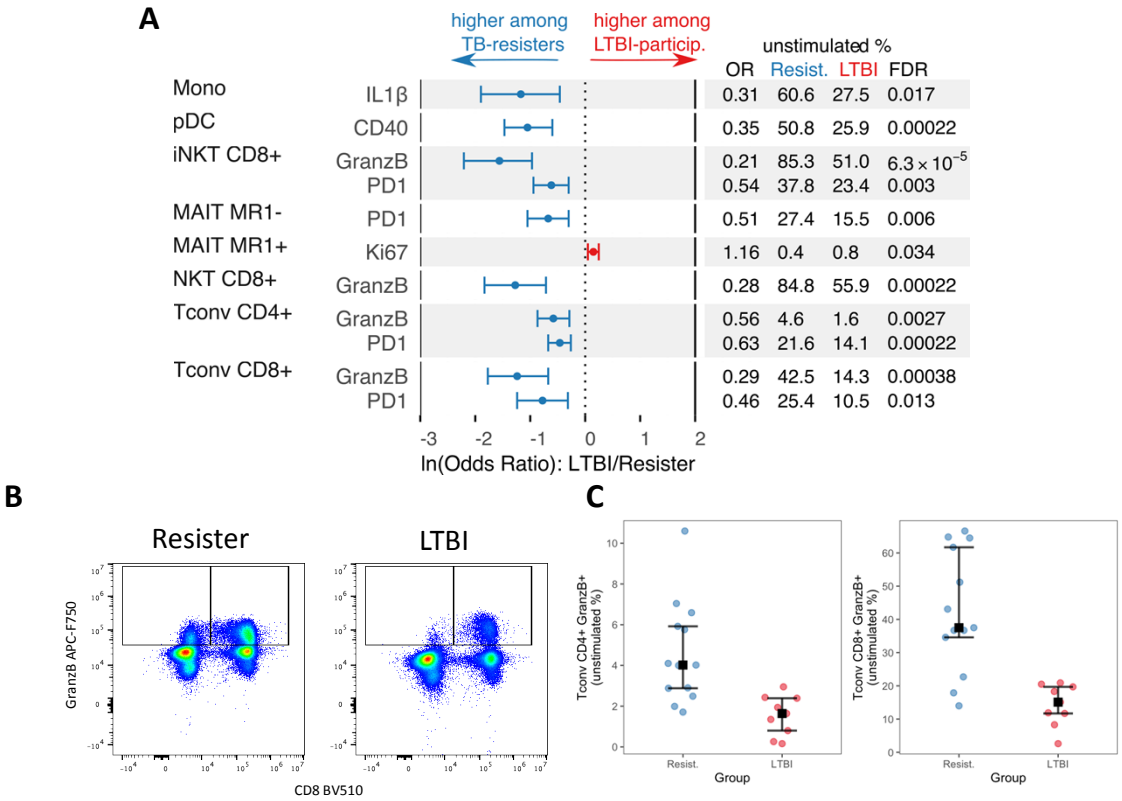



**Figure 1. Functional phenotypes differentially expressed in unstimulated PBMC from TB-resisters and LTBI-participants.** Data were derived from 13 TB-resisters and 11 LTBI-participants. PBMC were analyzed after overnight rest in culture medium. **A**. Forest plot displays ln-transformed odds ratios (lnOR) and 95% confidence intervals (CI). The dotted line shows no average effect (lnOR = 0, corresponding to OR = 1). Features on the right side of this line are more highly expressed among LTBI-participants (OR > 1), and features on the left side are more highly expressed among TB-resisters (OR < 1). The table shows the absolute OR and the means of each parameter in TB-resisters and LTBI-participants. **B.** Typical scatter plots exemplifying differences in GranzB+ CD4+ and CD8+ Tconv between TB-resisters (blue dots) and LTBI-participants (red dots). **C**. Dot plots showing frequencies of GranzB+ CD4+ and CD8+ Tconv in all participants.

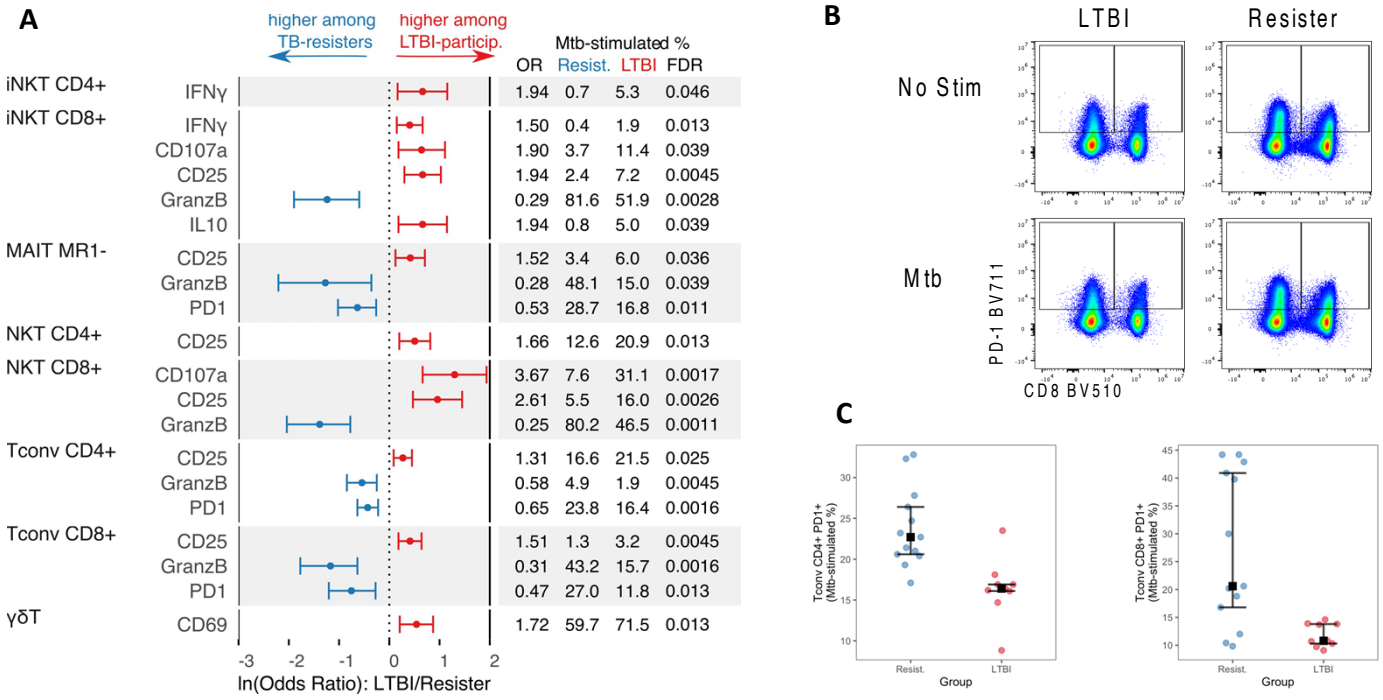



**Figure 2. Functional phenotypes differentially expressed in *Mtb*-stimulated PBMC from TB-resisters and LTBI-participants.** Data were derived from 13 TB-resisters and 9 LTBI-participants that contributed PBMC to the T cell analysis. PBMC were analyzed after pre-optimized overnight stimulation with *Mtb* cell membrane (see methods). **A**. Forest plot displays natural log (ln)-transformed odds ratios (lnOR) and 95% confidence intervals (CI). The dotted line shows no average effect (lnOR = 0, corresponding to OR = 1). Features on the right side of this line are more highly expressed among LTBI-participants (OR > 1), and features on the left side are more highly expressed among TB-resisters (OR < 1). The table shows the absolute OR and the means of each parameter in TB-resisters and LTBI-participants. **B.** Typical scatter plots exemplifying differences in PD1+ CD4+ and CD8+ Tconv between TB-resisters and LTBI-participants. **C**. Frequencies of GranzB+ CD4+ and CD8+ Tconv in all participants, including medians, upper and lower quartiles marked on the graph.

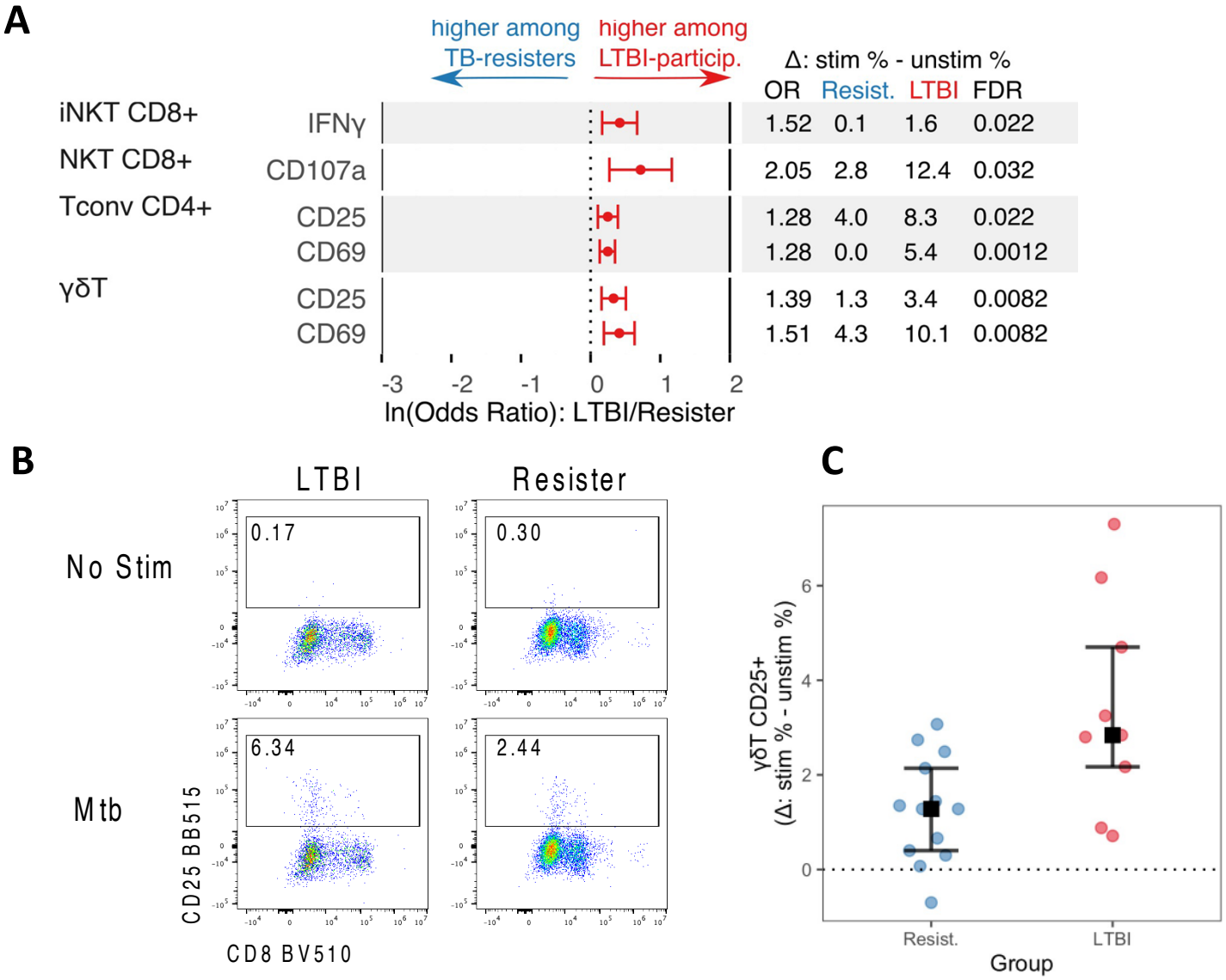
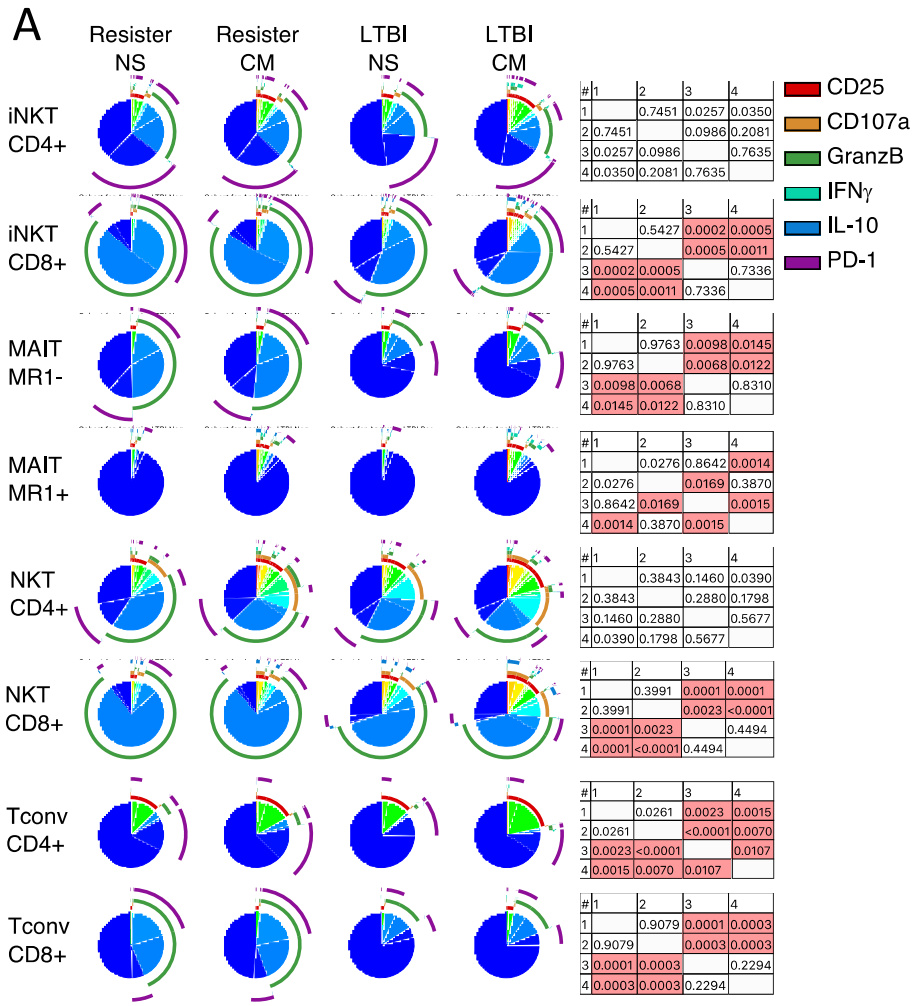



**Figure 3. *Mtb*-specific memory responses in TB-resisters and LTBI-participants.** Data were derived from 13 TB-resisters and 9 LTBI-participants that contributed PBMC to the T cell analysis. *Mtb*-memory was assessed by subtracting the frequency of activated cell subsets inn unstimulated PBMC from *Mtb*-stimulated PBMC. **A**. Forest plot displays ln-transformed odds ratios (lnOR) and 95% confidence intervals (CI) of the differences mentioned above. The dotted line shows no average effect (lnOR = 0, corresponding to OR = 1). Features on the right side of this line are more highly expressed among LTBI-participants (OR > 1), and features on the left side are more highly expressed among TB-resisters (OR < 1). The table shows the absolute OR

853 and the means of each parameter in TB-resisters and LTBI-participants. **B.** Typical scatter plots
854 exemplifying differences in *Mtb*-memory CD8+IFNγ+ iNKT and between TB-resisters and LTBI-
855 participants. **C**. Frequencies of CD8+IFNγ+ iNKT in all participants, including medians, upper and
856 lower quartiles marked on the graph.
857




| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.7451 | 0.0257 | 0.0350 |
| 2 | 0.7451 | | 0.0986 | 0.2081 |
| 3 | 0.0257 | 0.0986 | | 0.7635 |
| 4 | 0.0350 | 0.2081 | 0.7635 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.5427 | 0.0002 | 0.0005 |
| 2 | 0.5427 | | 0.0005 | 0.0011 |
| 3 | 0.0002 | 0.0005 | | 0.7336 |
| 4 | 0.0005 | 0.0011 | 0.7336 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.9763 | 0.0098 | 0.0145 |
| 2 | 0.9763 | | 0.0068 | 0.0122 |
| 3 | 0.0098 | 0.0068 | | 0.8310 |
| 4 | 0.0145 | 0.0122 | 0.8310 | |

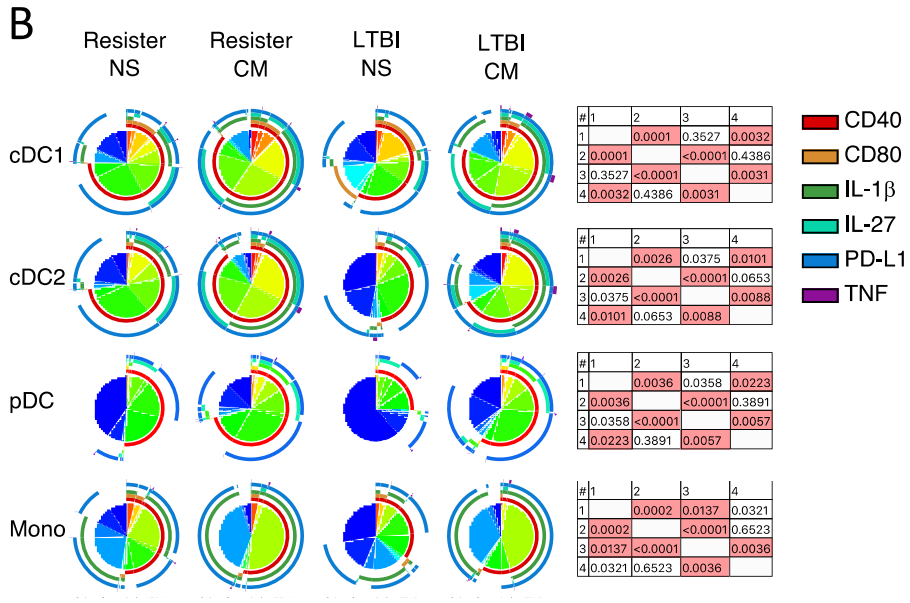
| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0276 | 0.8642 | 0.0014 |
| 2 | 0.0276 | | 0.0169 | 0.3870 |
| 3 | 0.8642 | 0.0169 | | 0.0015 |
| 4 | 0.0014 | 0.3870 | 0.0015 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.3843 | 0.1460 | 0.0390 |
| 2 | 0.3843 | | 0.2880 | 0.1798 |
| 3 | 0.1460 | 0.2880 | | 0.5677 |
| 4 | 0.0390 | 0.1798 | 0.5677 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.3991 | 0.0001 | 0.0001 |
| 2 | 0.3991 | | 0.0023 | <0.0001 |
| 3 | 0.0001 | 0.0023 | | 0.4494 |
| 4 | 0.0001 | <0.0001 | 0.4494 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0261 | 0.0023 | 0.0015 |
| 2 | 0.0261 | | <0.0001 | 0.0070 |
| 3 | 0.0023 | <0.0001 | | 0.0107 |
| 4 | 0.0015 | 0.0070 | 0.0107 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.9079 | 0.0001 | 0.0003 |
| 2 | 0.9079 | | 0.0003 | 0.0003 |
| 3 | 0.0001 | 0.0003 | | 0.2294 |
| 4 | 0.0003 | 0.0003 | 0.2294 | |




| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0001 | 0.3527 | 0.0032 |
| 2 | 0.0001 | | <0.0001 | 0.4386 |
| 3 | 0.3527 | <0.0001 | | 0.0031 |
| 4 | 0.0032 | 0.4386 | 0.0031 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0026 | 0.0375 | 0.0101 |
| 2 | 0.0026 | | <0.0001 | 0.0653 |
| 3 | 0.0375 | <0.0001 | | 0.0088 |
| 4 | 0.0101 | 0.0653 | 0.0088 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0036 | 0.0358 | 0.0223 |
| 2 | 0.0036 | | <0.0001 | 0.3891 |
| 3 | 0.0358 | <0.0001 | | 0.0057 |
| 4 | 0.0223 | 0.3891 | 0.0057 | |

| # | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| 1 | | 0.0002 | 0.0137 | 0.0321 |
| 2 | 0.0002 | | <0.0001 | 0.6523 |
| 3 | 0.0137 | <0.0001 | | 0.0036 |
| 4 | 0.0321 | 0.6523 | 0.0036 | |

858

859 **Figure 4. Polyfunctionality of immune responses in TB-resisters and LTBI-participants.**
860 Data were derived from 13 TB-resisters and 11 LTBI-participants. Pie charts 1-4 show proportions
861 of cells expressing no markers (dark blue) or ≥1 marker (other colors) in response to *Mtb*
862 stimulation or unstimulated control in the T cell panel (**A**) and APC panel (**B**). Arches show the
863 markers expressed by the responding cells. The permutation tables present p-values for
864 comparisons across TB-resisters unstimulated (1), TB-resisters *Mtb*-stimulated (2), LTBI-
865 participants unstimulated (3) and LTBI-participants *Mtb*-stimulated (4) conditions. Tables show
866 unadjusted p-values for each comparison. Pink boxes indicate significant differences defined by
867 FDR $p < 0.05$.
868

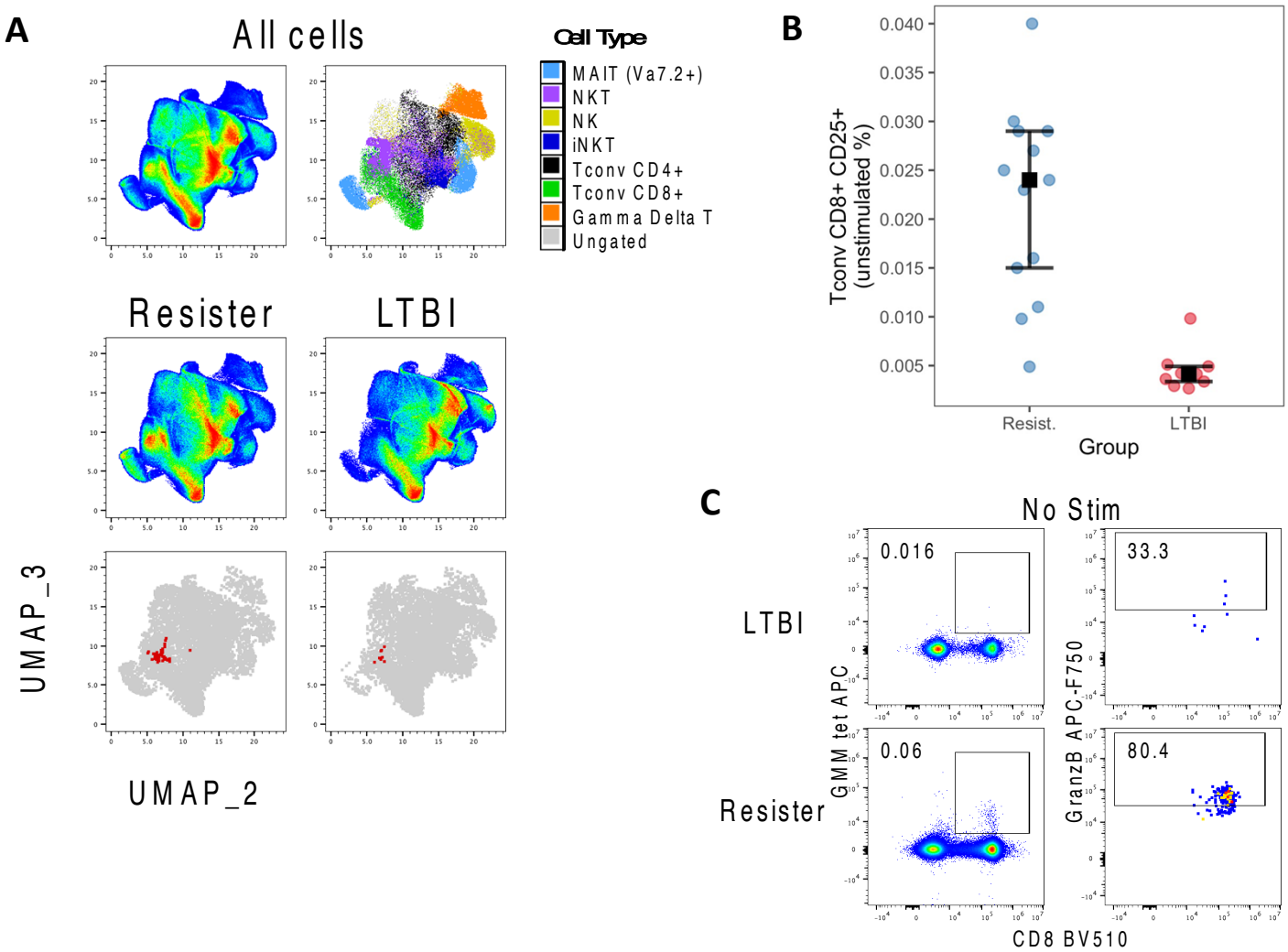



**Figure 5. Comparison of CD8+GMM+GranzB+ Tconv subset in TB-resisters and LTBI-participants.** Data were derived from 13 TB-resisters and 9 LTBI-participants that contributed 2,948,000 events down sampled to 67,000 events in the cleanup gate. **A**: T-cell UMAPs showing concatenated stimulated and unstimulated cells (upper left corner), distribution of major cell subsets (upper right corner); Distribution of unstimulated T cells in Resisters and LTBI-participants (middle row); CD8+GMM+GranzB+ cluster in Resister and LTBI-participants (bottom row). Representation of typical scatter plot. **B**: Individual results from each participant with medians,

879 upper and lower quartiles are identified on the graph. **C:** Typical representation of the frequency

880 of CD8+GMM+GranzB+ T cells in LTBI-participants and Resisters.

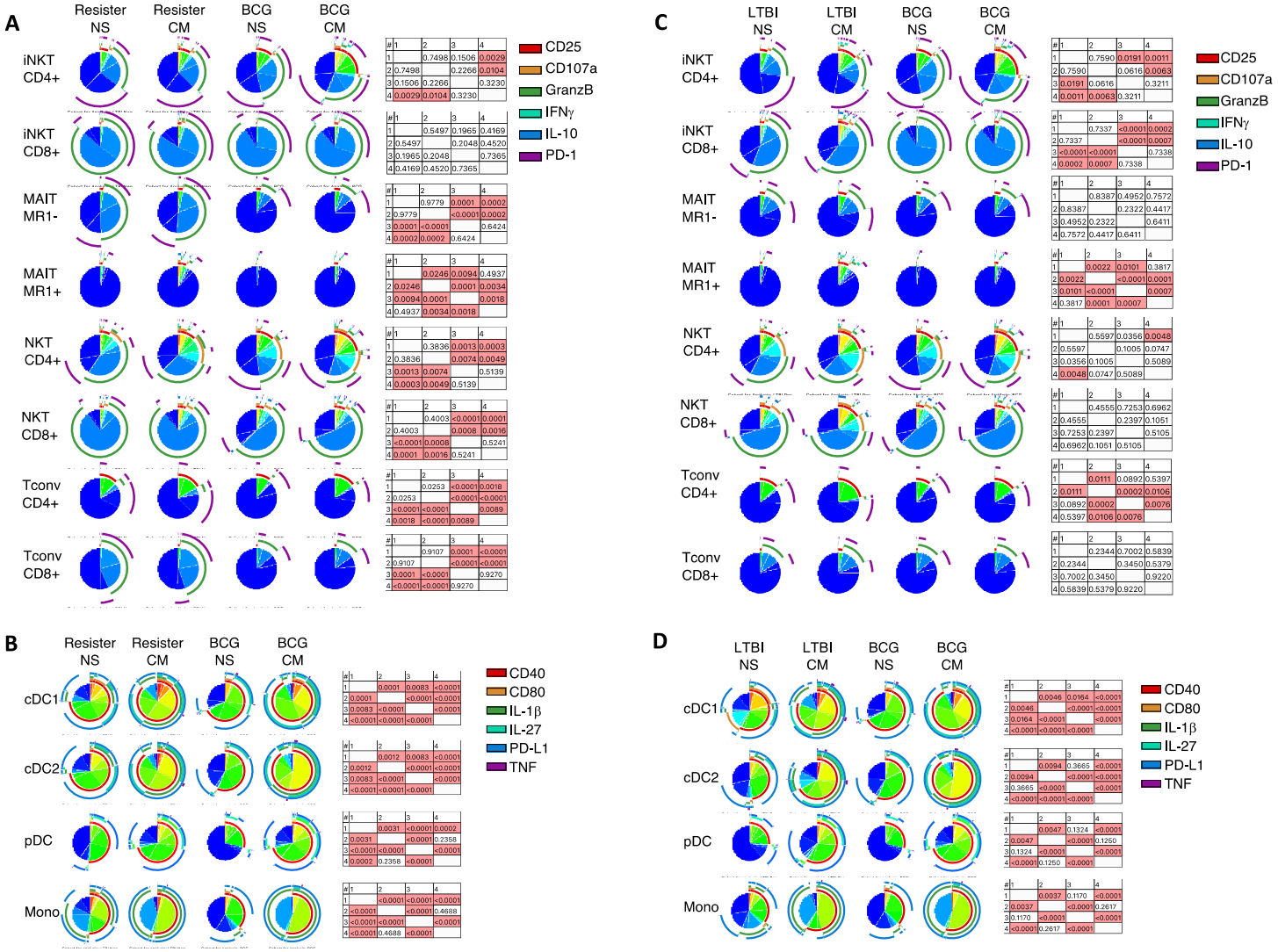



**Figure 6. Polyfunctionality of immune responses in BCG-recipients by comparison with TB-resisters and LTBI-participants.** Data were derived from 14 BCG-recipients, 13 TB-resisters, and 11 LTBI-participants. Pie charts 1-4 show proportions of cells expressing no markers (dark blue) or ≥1 marker (other colors) in response to *Mtb* stimulation or unstimulated control. Arches show the markers expressed by the responding cells. The permutation tables show unadjusted p-values for each comparison. Pink boxes indicate significant differences defined by FDR p < 0.05). **A:** T cell panel in BCG-recipients and TB-resisters; **B**: APC panel in BCG-recipients and TB-resisters; C: T cell panel in BCG-recipients and LTBI-participants; **D**: APC

891 panel in BCG-recipients and LTBI-participants. The T cell panels show selected subsets with
892 significant differences of BCG-recipients with TB-resisters and/or LTBI-participants defined by
893 FDR $p<0.05$. Remaining subsets are shown in **Fig S6**.