1 **Title**

2 Polyfunctional CD4 T-cells correlate with *in vitro* mycobacterial growth inhibition following

3 *Mycobacterium bovis* BCG-vaccination of infants

4

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17

18 **Abbreviations:** Bacillus Calmette-Guerin (BCG); purified protein derivative (PPD);

19 Staphylococcus enterotoxin B (SEB); tuberculosis (TB); non-tuberculous mycobacteria

20 (NTM); interquartile range (IQR); intracellular cytokine staining (ICS); mycobacterial

21 growth inhibition assay (MGIA)

22

23

24

25 **Abstract**

26 **Background:** Vaccination with Bacillus Calmette Guerin (BCG) protects infants against
27 childhood tuberculosis however the immune mechanisms involved are not well understood.
28 Further elucidation of the infant immune response to BCG will aid with the identification of
29 immune correlates of protection against tuberculosis and with the design of new improved
30 vaccines. The purpose of this study was to investigate BCG-induced CD4+ T-cell responses
31 in blood samples from infants for cytokine secretion profiles thought to be important for
32 protection against tuberculosis and compare these to PBMC-mediated *in vitro* mycobacterial
33 growth inhibition. **Methods:** Blood from BCG-vaccinated or unvaccinated infants was
34 stimulated overnight with *Mycobacterium tuberculosis* (*M. tb*) purified protein derivative
35 (PPD) or controls and intracellular cytokine staining and flow cytometry used to measure
36 CD4+ T-cell responses. PBMC cryopreserved at the time of sample collection were thawed
37 and incubated with live BCG for four days following which inhibition of BCG growth was
38 determined. **Results:** PPD-specific IFNγ+TNFα+IL-2+ CD4+ T-cells represented the
39 dominant T-cell response at 4 months and 1 year after infant BCG. These responses were
40 undetectable in age-matched unvaccinated infants. IL-17+ CD4+ T-cells were significantly
41 more frequent in vaccinated infants at 4 months but not at 1-year post-BCG. PBMC-mediated
42 inhibition of mycobacterial growth was significantly enhanced at 4 months post-BCG as
43 compared to unvaccinated controls. In an analysis of all samples with both datasets available,
44 mycobacterial growth inhibition correlated significantly with the frequency of polyfunctional
45 (IFNγ+TNFα+IL-2+) CD4+ T-cells. **Conclusions:** These data suggest that BCG vaccination
46 of infants induces specific polyfunctional T-helper-1 and T-helper-17 responses and the
47 ability, in the PBMC compartment, to inhibit the growth of mycobacteria in *vitro.* We also
48 demonstrate that polyfunctional T-helper-1 cells may play a role in growth inhibition as
49 evidenced by a significant correlation between the two.

50

51 **Keywords:** BCG; T-cells; Cytokines; Infants; Polyfunctional; Mycobacterial Growth

52 Inhibition

53 **Introduction**

54 A major challenge in the field of tuberculosis (TB) vaccinology is the need for a better
55 understanding of how Bacillus Calmette-Guerin (BCG), the only licensed vaccine in use
56 against TB, confers protection in some populations and settings but not others [1-3].
57 Knowledge of the mechanisms underlying this difference is vital for the ongoing search for a
58 better vaccine. One TB vaccine candidate that had proved efficacious in pre-clinical animal
59 models [4,5] did not give infants significant protection from TB disease [6]. In that clinical
60 trial, the vaccine, MVA85A, was administered to infants already vaccinated at birth with
61 BCG with the aim of enhancing any BCG-mediated protection. That this did not occur has
62 prompted the suggestion that the protection afforded by BCG alone may be difficult to
63 improve upon and as such, its mechanisms of action deserve greater attention if this is to
64 happen [7].

65 Our group has previously provided an immunological description of the different responses
66 to BCG in United Kingdom (UK) and African populations in which the efficacy of BCG
67 differs. Malawian adolescents displayed high baseline immune responses to mycobacterial
68 antigen and BCG did not enhance these. In the UK, adolescents displayed low baseline
69 responses which were increased significantly following BCG [8]. These observations were
70 consistent with the hypothesis that exposure to non-tuberculous mycobacteria (NTM) was
71 masking the response to BCG [9]. In subsequent reports we expanded this description to
72 infants where, although pre-exposure to NTM was unlikely given the very young age of the
73 study group, differences in immune response were still observed between the two settings.
74 UK infants made stronger T helper (Th)-1-type responses including IFNγ whereas Malawian
75 infants made stronger Th-2 and regulatory-type responses [10]. Although Th-1 responses,
76 including cytokines such as IFNγ and antigen-specific CD4+ T-cells, have been reported not
77 to correlate with protection against, or risk of, TB disease [11-15], they remain a focus of

78 interest for TB vaccinologists due to contrasting evidence where these cells do seem to
79 correlate with protection against TB [16] as well as the strong evidence in favour of their
80 importance based on studies of patients with IFNγ signaling deficiencies [17,18] or with HIV
81 infection and low CD4 counts [19] who are both susceptible to TB.

82 A recent development in the field of TB biomarker discovery has been the establishment of
83 techniques that allow the measurement of mycobacterial growth inhibition *in vitro*. This
84 assessment of the capacity of a compartment such as whole blood or peripheral blood
85 mononuclear cells (PBMC) to inhibit the growth of mycobacteria represents an unbiased
86 surrogate marker of protection against TB and allows for investigations into the potential
87 roles of immune mechanisms of interest in mediating this "protective" effect [20-23].

88 In light of the recent evidence [24] that BCG vaccination in high latitude, low NTM settings
89 such as the UK might provide important information regarding BCG-induced protective
90 immunity, we hypothesized that the cytokine immune responses we previously described in
91 UK infants would originate from antigen-specific helper T-cells. We report here our findings
92 that BCG vaccination does indeed induce long lasting, polyfunctional Th-1 cellular responses
93 as well as a more transient Th-17 response in UK infants. We also show that BCG
94 vaccination induces PBMC-mediated mycobacterial growth inhibition and that
95 polyfunctional Th-1 responses may play a role in this.

96 **Materials and Methods**

97 *Study participants and sample collection*

98 Healthy infants, born in the UK to mothers with no history of chronic illness including HIV
99 infection, were recruited after written informed consent was obtained from parents.
100 Depending upon local health service policy, infants either received a single dose of
101 intradermal BCG (BCG Vaccine Danish Strain 1331, Staten Serum Institute, Copenhagen,
102 Denmark) at community clinics (median vaccination age: 5.6 weeks, interquartile range
103 (IQR): 4.3-8.0 weeks) or did not receive BCG. Heparinised venous blood samples were
104 obtained from infants at two time points selected to provide data at distinct intervals (4
105 months and 1 year) following vaccination or at age-matched time points in unvaccinated
106 infants. Due to variations in availabilities of infant participants and parents, actual median
107 sampling time points were 15.3 weeks (IQR: 14.4-18.0) and 56.3 weeks (IQR: 54.9-58.0).
108 Ethical approval for the study was granted by the National Research Ethics Service
109 Committee London-East (11/LO/0363) and by the Ethics Committee of the London School of
110 Hygiene and Tropical Medicine.

111

112 *Diluted whole blood intracellular cytokine staining (ICS) assay*

113 Venous blood (0.5 mL) was diluted 1:1 with warm Iscove's Modified Dulbecco's Medium
114 (Lonza, Belgium) in 15 mL centrifuge tubes (Corning Inc. NY). Diluted blood was incubated
115 alone (medium only negative control), with 10 µg/ml *M. tb* PPD batch RT50 for *in vitro* use;
116 Statens Serum Institute, Copenhagen, Denmark) or with 5 µg/ml staphylococcus enterotoxin
117 B (SEB; Sigma, UK) as a positive control. Co-stimulatory antibodies (2 µg/ml each of anti-
118 CD28 and anti-CD49d (BD Biosciences, Oxford, UK)[25]) were added to all antigen and
119 control tubes. Assay tubes were incubated with loose lids for 2 hours at 37°C after which 3
120 µg/ml of brefeldin A (Sigma, UK) was added to all tubes which were then incubated for a

121 further 18 hours at 37°C. The average time between venipuncture and the initiation of ICS
122 assay stimulations was 3 hours 16 minutes (SD: 56 minutes). Following incubations diluted
123 blood was incubated with 10 volumes of 1x PharmLyse solution (BD Biosciences, Oxford,
124 UK) at room temperature for 10 minutes to lyse red blood cells, centrifuged and washed with
125 3 mL PBS. Prior to permeabilisation, pelleted cells were surface stained with Vivid live/dead
126 reagent (Molecular Probes), anti-CD4-APC-H7 (BD Biosciences), anti-CD19-efluor450 and
127 anti-CD14-efluor450 (eBiosciences) for 30 minutes at 4°C. After washing in PBS + 0.1%
128 BSA + 0.01% sodium azide, cells were permeabilised with Cytofix/Cytoperm reagent (BD
129 Biosciences) at 4°C for 20 minutes, washed in Perm Wash buffer (BD Biosciences) and
130 stained with anti-CD3-Horizon-V500, anti-IL-2-FITC, anti-TNFα-PE-Cy7 (BD Biosciences),
131 anti-IL-17-efluor660, anti-IL-10-PE (eBiosciences) and anti-IFNγ-PerCP-Cy5.5 (Biolegend)
132 for 30 minutes at room temperature. Cells were finally resuspended in 250 μL 1%
133 paraformaldehyde (Sigma, UK) and filtered prior to acquisition. Data was acquired using an
134 LSRII flow cytometer (BD Biosciences) configured with 3 lasers and 10 detectors and
135 FACSDiva acquisition software (BD Biosciences). Compensation was performed using tubes
136 of CompBeads (BD Biosciences) individually stained with each fluorophor and
137 compensation matrices were calculated with FACSDiva.
138
139 *PBMC cryopreservation and* in vitro *mycobacterial growth inhibition assay*
140 For infant blood samples where enough material was available following whole blood ICS
141 assays, PBMC were prepared by density centrifugation and cryopreserved. Briefly, blood
142 diluted 1/3 in HBSS (Invitrogen) was layered over Histopaque 1077 (Sigma) and centrifuged
143 at 800g for 20 minutes. The PBMC layer was transferred to a fresh tube, washed 3 times with
144 HBSS, frozen in RPMI 1640 (Invitrogen) with 20% foetal bovine serum (FBS; Invitrogen)
145 and 10% dimethylsulfoxide (Sigma) and stored at -80°C for 24 hours before transfer to liquid

146 nitrogen. On thawing, cells were rested for two hours at 37°C in RPMI 1640 with 10% FBS
147 and 10 units/ml of benzonase (Novagen), then washed and re-suspended in RPMI 1640 with
148 25 mM HEPES (Sigma) supplemented with 2 mM L-glutamine and 10% filtered, heat-
149 inactivated, pooled human AB serum (Sigma). PBMC ($1x10^6$) were added to 2ml screw-cap
150 microtubes (Sarstedt, Germany) with a pre-determined, optimal quantity of BCG Danish that
151 equated to 862 cfu and made up to a final volume of 600 µl. Tubes were incubated at 37°C
152 with 360° rotation for 96 hours. Following incubations, cells and remaining BCG were
153 pelleted and cells lysed by incubation in sterile water with vortexing. BCG from a single tube
154 were then transferred into a corresponding MGIT tube and time to positivity determined
155 using a MGIT 960 (Becton Dickinson). As all assays were carried out simultaneously in a
156 single batch, direct-to-MGIT controls were not used, as the calculation of relative growth was
157 not required.
158
159 *Data analysis, management and statistical analysis*
160 ICS flow cytometric data was analysed using FlowJo software version 9 (TreeStar Inc.) and
161 Spice version 5 [26]. Samples were gated sequentially on singlet, live, CD14-CD19-,
162 lymphoid, CD3+CD4+ cells and negative control stimulation tubes were used to set cytokine
163 gates (Supplementary Fig. 1). Median cytokine responses in negative control tubes, as a
164 percentage of the gated CD4+ T-cell population, were as follows: IFNγ – 0.003%; TNFα –
165 0.002%; IL-2 – 0.002%; IL-17 – 0.002%; IL-10 – 0.003%. Median cytokine responses in
166 positive control tubes (SEB-stimulated) were as follows: IFNγ – 0.16%; TNFα – 2.05%; IL-2
167 – 1.77%; IL-17 – 0.11%; IL-10 – 0.06% (Supplementary Fig. 2). Cytokine responses reported
168 for all stimuli are after subtraction of background values measured in un-stimulated tubes.
169 The median number of CD4+ T-cell events acquired for all tubes was 298,895 (IQR: 239875-
170 312526).

171 All mycobacteria growth inhibition assays were carried out in duplicate. For each tube, time
172 to positivity in hours was converted to log CFU of bacteria using a previously determined
173 growth curve for the stock of BCG used.
174 All statistical comparisons between the magnitudes of ICS and growth inhibition responses
175 between vaccinated and unvaccinated groups were made using the Mann-Whitney U test.
176 Associations between growth inhibition and ICS responses were measured using Spearman's
177 rank correlation coefficient.

178 **Results**

179 *BCG-vaccination of infants at 6 weeks of age induces antigen-specific, polyfunctional Th-1*
180 *cells that persist up to at least 1 year post-vaccination*

181 In order to assess the ability of infant BCG vaccination to activate cytokine-secreting helper
182 T-cells we used a diluted whole blood ICS assay and PPD stimulation to measure the
183 frequency of mycobacteria antigen-specific CD4+ T-cell responders at an early and a later
184 time point following vaccination. CD4+ T-cells expressing IFNγ, TNFα and IL-2 were
185 detectable at 4 months and 1 year after vaccination but not in age-matched samples from
186 unvaccinated infants (Fig. 1A-C). The application of Boolean gating to determine the pattern
187 of cytokine co-expression revealed that at both 4 months and 1 year, IFNγ+TNFα+IL-2+
188 polyfunctional T-cells formed the dominant responder population in BCG-vaccinated infants.
189 The overall responder profile, which also included TNFα+IL-2+ double positive and TNFα+
190 single positive populations, was almost identical at both time points after vaccination (Fig.
191 1D). These data support the conclusion that BCG vaccination of UK infants activates an
192 antigen-specific, polyfunctional Th-1 response that is still detectable one year after
193 vaccination.

194

195 *Antigen-specific Th-17 cells are increased in vaccinated infants at 4 months post-vaccination*
196 *compared to unvaccinated controls*

197 In addition to IFNγ, TNFα and IL-2, we also examined CD4+ T-cells for antigen-specific,
198 IL-17 and IL-10 responses. Although we could not detect any IL-10 response with this
199 protocol at either time point (data not shown), we did observe a significantly greater
200 frequency of IL-17+ CD4+ T-cells in vaccinated compared to unvaccinated infants at 4
201 months (median responses of 0.011% IL-17+ and 0.002% IL-17+ CD4 T-cell respectively;
202 $p=0.0045$) but not at 1 year (median responses of 0.007% IL-17+ and 0.006% IL-17+ CD4 T-

203 cell respectively; p=0.894; Fig. 2A-C). In order to determine whether IL-17+ CD4+ T-cells
204 were secreting other cytokines, we restricted analysis to all possible IL-17+ signatures (Fig.
205 2D), which revealed that the dominant secretion profile was IL-17+ single positive events.
206 Although the response profile was similar at both time points, there was a significant
207 reduction in the frequency of two less prominent populations at 1 year, namely IL-
208 17+TNFα+IL-2+ triple positive and IL-17+IL-2+ double positive cells (p=0.03 and p=0.009
209 respectively; Fig. 2D). These data support the conclusion that BCG vaccination of UK infants
210 activates an antigen-specific Th-17 response that, although prominent at 4 months post-BCG,
211 at 1-year post-BCG has reduced to a magnitude that is indistinguishable from that seen in
212 unvaccinated infants.
213

214 *PBMC compartment of vaccinated infants displays an increased capacity to inhibit the* in
215 vitro *growth of BCG mycobacteria at 4 months post-vaccination*

216 In parallel to diluted whole blood ICS assays, where sample volume permitted, PBMC were
217 also prepared from infant blood samples and cryopreserved. These PBMC were later thawed
218 and tested for the capacity to inhibit mycobacterial growth in *vitro* with an assay used as a
219 surrogate marker for protective immune responses against *Mycobacterium tuberculosis in*
220 *vivo*. Growth of BCG mycobacteria was significantly inhibited by PBMC from BCG-
221 vaccinated infants at 4 months. The median log CFU measured after 4-day culture was 3.2 as
222 compared to 5.1 in unvaccinated infants (p<0.001; Fig. 3A). The difference in median log
223 CFU at 12 months post-vaccination between vaccinated and age-matched unvaccinated
224 infants however was not significant (4.2 and 5.1 respectively; p=0.294; Fig. 3B). As samples
225 from vaccinated and unvaccinated infants at both time points exhibited a range of log CFU
226 values that represented the varying degrees of mycobacterial inhibition, we compared these
227 data with matching frequencies of polyfunctional (IFNγ+TNFα+IL-2+) and Th-17 (IL-17+)

228 CD4 T-cells for those samples where both MGIA and ICS data were available. In order to
229 avoid including dependent variables in the correlation analysis, where data was available for
230 one infant at both time points, only the 4-month data was included. There was a significant
231 degree of inverse correlation between polyfunctional CD4+ T-cell frequency and log CFU
232 (Spearman r value = -0.709; p=0.013; Fig. 3C) but not between Th-17 CD4 T-cell frequency
233 and log CFU (Spearman r value = -0.399; p=0.198; Fig. 3D) as measured after growth
234 inhibition assays.
235 In summary, BCG vaccination of UK infants induces polyfunctional and Th-17 CD4 T-cells
236 and bestows upon the PBMC compartment the capacity to inhibit mycobacterial growth.
237 Further to these observations, there is an association between the magnitude of the
238 polyfunctional T-cell response and the level of mycobacterial inhibition detected *in vitro*.

239 **Discussion**

240 We have measured a population of PPD-specific, CD4+ T-cells that demonstrates
241 polyfunctional (IFNγ+TNFα+IL-2+) capabilities in infants who have received BCG
242 vaccination at 6 weeks of age. This population was detectable at 4 months and 1 year post-
243 vaccination, comprised the most abundant population as compared to other possible secretion
244 profiles at both time points and was absent in unvaccinated infants. This result complements
245 previous immune analyses we have carried out on similar infant cohorts where secreted
246 cytokine responses, including IFNγ, TNFα and IL-2, were observed in vaccinated but not
247 unvaccinated infants following PPD stimulation of diluted whole blood [27,28], and partly
248 answers the question as to which cells were secreting cytokines in those studies. A number of
249 papers have consistently described a more heterogeneous T-helper response in terms of the
250 cytokine secretion profiles detected following BCG in infants [15,29-31]. Although
251 IFNγ+TNFα+IL-2+ triple positive cells are usually detectable in those studies, it is often
252 single cytokine producers, most notably IFNγ+ Th-1 cells, which are most frequent. In
253 contrast, we did not detect any single IFNγ producers and in fact, the only population that
254 displayed any IFNγ positivity was expressing all three cytokines. The studies referred to
255 above were carried out in Africa, therefore it is possible that these differences in response
256 could be due to the fact that our study was carried out in a northern European setting and so
257 infant participants comprised a different ethnic population who were exposed to a contrasting
258 set of environmental factors. Another possible explanation for the difference between these
259 results and those from the African settings could be differences in stimulation protocols, most
260 notably the use of live BCG mycobacteria as a stimulant in the African studies and our use of
261 PPD. In addition to this study, others who have used PPD as a stimulant to investigate T-cell
262 responses following BCG vaccination have also reported prominent polyfunctional responses
263 in mice and humans [32,33].

264 Clearly, the implication of observing a persistent, polyfunctional population of Th-1 cells that
265 is very specific to having received the BCG vaccine, is that these cells are in some way
266 involved in the protection that BCG affords these infants against childhood TB [2]. One of
267 the first flow cytometric descriptions of polyfunctional, cytokine-producing T-cells was in
268 the context of the protection they conferred to mice following efficacious vaccination against
269 *Leishmania major* [34]. The field of TB has produced mixed data regarding the role of
270 polyfunctional cells in immune protection. Retrospective analysis of samples taken 10 weeks
271 after BCG vaccination at birth showed no difference in polyfunctional Th-1 cell frequency
272 when infants who eventually contracted TB disease were compared to those who were
273 exposed but remained disease-free during a 2 year follow-up period [15]. Another recent
274 example of polyfunctional Th-1 cells failing to bestow protection against TB was when no
275 significant protective effect was demonstrated following administration of novel TB vaccine
276 MVA85A to infants despite this vaccine's ability to boost antigen-specific, IFNγ+TNFα+IL-
277 2+ CD4 T-cells [6]. Evidently, polyfunctional Th-1 cells do not correlate with risk of TB
278 disease or protection, respectively, in these studies carried out in African settings, however,
279 there are other circumstances where polyfunctional T-cell responses are associated with
280 groups who have proved resistant to the development of TB disease; namely HIV-positive
281 patients after anti-retroviral therapy with higher CD4 counts or those with lower viral loads
282 [35,36]. The relatively low TB incidence in the UK combined with the design of our study
283 means it is not possible to determine whether the Th-1 population we describe here correlates
284 inversely or directly with risk or protection respectively, although it seems that the field
285 would benefit greatly from an assessment of the protective capacity of polyfunctional T-cells
286 in a low NTM, high latitude setting.

287 In mice, IL-17 responses in the lung were shown to precede an influx of protective Th-1 cells
288 following vaccination and subsequent infection with *M. tb* [37] and furthermore, the IL-

289 23/IL-17 pathway was necessary following BCG vaccination for the effective generation of a
290 Th-1 response specifically to overcome an otherwise inhibitory IL-10 response [38]. In
291 addition to polyfunctional Th-1 cells, we also report here a population of PPD-specific, IL-
292 17+CD4+CD3+ cells that are present at a significantly greater frequency in BCG-vaccinated
293 infants at 4 months post-BCG, as compared to age-matched, unvaccinated infants. Again, this
294 is consistent with our previous reports of PPD-specific, IL-17 responses detectable in BCG-
295 vaccinated infants [27,28]. The current data provide evidence that the source of at least some
296 of this IL-17 is antigen-specific Th-17 cells. Unlike Th-1 cellular profiles, Th-17 cells have
297 been less frequently reported in infant BCG studies, partly due to the more recent emergence
298 of IL-17 as a cytokine of interest. Where a Th-17 response was sought, in one report it was
299 not detectable above background levels in control stimulations [39], however in others it was
300 detectable but did not correlate with risk of TB disease in South African infants [15], nor did
301 it differ in Ugandan infants who received BCG at birth as compared to those who received it
302 at 6 weeks of age [31]. We have previously described the complete absence of a PPD-
303 specific, secreted IFNγ response in unvaccinated UK infants up to 15 months of age [40] and
304 describe here a concomitant absence of IFNγ+ Th-1 cells in a similar cohort. This very low
305 background immune responsiveness to PPD extends to IL-17 and has allowed us to
306 distinguish an otherwise low frequency Th-17 response at 4 months in vaccinated infants.
307 However, this differential response at 4 months post-BCG did not extend to samples taken at
308 1-year post-BCG. It is interesting to note that whilst the frequency of the Th-17 response in
309 BCG-vaccinated infants is lower at 12 months than at 4 months, the inverse is true of
310 unvaccinated infants. It has been shown that there is a strong bias towards the development of
311 Th-17 in neonates [41]. In our study, it may be that this propensity is being illustrated in two
312 ways. Firstly, in the large increase in PPD-specific Th-17 responses due to BCG vaccination
313 that gradually wanes by 12 months. Secondly, in the less pronounced increase in Th-17

314 responses in BCG unvaccinated infants. That the latter responses were stimulated by PPD
315 suggests that either these infants have been exposed to environmental mycobacteria or that
316 there is some cross-reactivity in these Th-17 responses.
317 We did not detect any IL-10 responses in this study. Although we previously detected PPD-
318 induced IL-10 in BCG vaccinated infants it was in diluted whole blood culture supernatants
319 after 7 days using multiplex bead array. [27,28]. In this study we used a much shorter
320 stimulation period (18 hours) and ICS as a detection method and these may be a less sensitive
321 than is required for IL-10. Another possible explanation is that we restricted our analysis to
322 $CD4^+$ T-cells in this study and may be missing IL-10 responses from cells such as
323 monocytes.
324 In the absence of a TB disease or infection outcome in our cohort we employed instead an *in*
325 *vitro* mycobacteria growth inhibition assay (MGIA) as a surrogate marker of immune
326 resistance. There is much interest in developing functional assays such as the MGIA as a
327 surrogate marker of protection in order to facilitate the testing of novel TB vaccines. The aim
328 is that the MGIA will eventually circumvent the need for long, expensive follow up periods
329 with TB disease or infection as an endpoint. An additional advantage is that the unbiased
330 nature of the MGIA means that prior knowledge as to which component of the immune
331 system is responsible for any protective effect is not necessary. In this study, we found that
332 the PBMC compartment of vaccinated infants contained the ability to inhibit the growth of
333 BCG mycobacteria *in vitro* to a greater extent than that of unvaccinated infants in samples
334 taken at 4 months. However by 12-month post-BCG there was no difference between the
335 vaccinated and unvaccinated groups. A previous study has shown that PBMC from adults
336 with a history of BCG vaccination inhibit the growth of BCG mycobacteria *in vitro* to a
337 greater extent than PBMC from adults without a history of BCG vaccination and that the
338 capacity to inhibit mycobacterial growth can be induced in the BCG 'naïve' adults with

339 subsequent, primary vaccination [42]. In another study using a different version of the growth
340 inhibition assay involving luminescent BCG and whole blood samples, neonatal BCG
341 vaccination induced a significant degree of mycobacterial growth inhibition that persisted for
342 up to 6 months when compared to growth inhibition in pre-vaccination samples [43]. Based
343 on the data available, it is difficult to speculate as to why adults who have had BCG many
344 years previously may display significantly more growth inhibition than adults with no history
345 of BCG where as in this study, the difference between vaccinated and unvaccinated infants
346 lost statistical significance by 12 months post vaccination. The interquartile range of the 12-
347 month post-BCG growth inhibition data was much greater in the vaccinated group. This may
348 be due to the relatively small sample size and a bigger study with more infants might allow
349 smaller differences between groups to be detectable that are not here. It should be noted
350 however that in the Fletcher *at al* study [42], adults receiving primary BCG vaccination that
351 displayed significant PBMC-mediated growth inhibition at 4 weeks post-vaccination had
352 returned to baseline levels by 6 months post-vaccination which is more consistent with the
353 findings we present here.
354 The data presented here support the hypothesis that PPD-specific polyfunctional CD4 T-cells
355 secreting IFNγ, TNFα and IL-2 play a role in mycobacterial growth inhibition *in vitro*. It is
356 interesting that three previous studies [21,42,43] did not show any correlation between PPD-
357 specific IFNγ responses measured by ELISA or ELISpot and growth inhibition. To our
358 knowledge, this is the first time growth inhibition and polyfunctional CD4 T-cells have been
359 compared in infants and that a correlation is detected might indicate that measuring these
360 cells is revealing a mechanism that looking at IFNγ alone might be missing.
361 Our data involves a correlation between ICS responses measured in diluted whole blood and
362 MGIA responses measured in PBMC, two different cellular compartments. We cannot rule
363 out possible implications (and limitations) of this, for example, the presence of

364 polymorphonuclear cells in ICS assays and their absence in MGIA or the use of non-

365 autologous pooled human serum in MGIA. It would be worth, in future, repeating this

366 experiment using a whole blood version of the growth inhibition assay to complement the

367 whole blood ICS in order to investigate the possible impact of this.

368 In conclusion, the present study expands our previous description of cytokine and chemokine

369 immune responses in BCG vaccinated UK infants to reveal a prominent, long-lived and

370 mainly polyfunctional Th-1 response together with the induction of antigen-specific Th-17

371 cells and that the magnitude of polyfunctional Th-1 cells correlates with *in vitro*

372 mycobacterial growth inhibition and may play a role in this effect.

373 **Conflicts of interest: none**

374

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385

386

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541 **Figure legends**

542 **FIGURE 1. Persistent, polyfunctional T helper-1 cells are the dominant, PPD-specific**

543 **CD4+ T-cell population in BCG-vaccinated infants.**

544 Venous blood samples obtained 4 months and 1-year post-BCG from vaccinated infants or

545 from age-matched, unvaccinated infants were stimulated with PPD overnight and CD4+ T-

546 cells assessed for cytokine secretion by intracellular staining. An example of IFNγ, TNFα

547 and IL-2 co-staining is shown for 4 month (**A**) and 1 year (**B**) samples from a BCG

548 vaccinated (+) and an unvaccinated (−) infant and cumulative composite, single cytokine

549 expression data (**C**) for vaccinated and unvaccinated infants at 4 months (dark grey circles)

550 and 1 year (light grey circles). (**D**) The polyfunctionality of CD4+ T-cell cytokine responses

551 in BCG-vaccinated infants at 4 months (dark grey bars) and 1 year (light grey bars). All

552 composite data plots display boxes showing medians and interquartile ranges. Whiskers,

553 where included, show the data range. The Mann-Whitney U test was used to determine

554 significance. Sample numbers are n=24 (BCG-vaccinated infants at 4 months post-BCG);

555 n=18 (BCG-vaccinated infants at 1 year post-BCG); n=12 (BCG-unvaccinated infants at 4

556 month time point) and n=15 (BCG-unvaccinated infants at 1 year time point).

557

558 **FIGURE 2. PPD-specific T helper-17 response in BCG-vaccinated infants.**

559 An example of IL-17, IFNγ, TNFα and IL-2 co-staining is shown for 4 month (**A**) and 1 year

560 (**B**) samples from a vaccinated (+) and an unvaccinated (−) infant. (**C**) Cumulative,

561 composite IL-17 expression data for vaccinated and unvaccinated infants at 4 months (dark

562 grey circles) and 1 year (light grey circles). (**D**) The polyfunctionality of all IL-17-expressing

563 CD4+ T-cell phenotypes in BCG vaccinated infants at 4 months (dark grey bars) and 1 year

564 (light grey bars). Sample numbers are n=24 (BCG-vaccinated infants at 4 months post-BCG);

565 n=18 (BCG-vaccinated infants at 1 year post-BCG); n=12 (BCG-unvaccinated infants at 4

566 month time point) and n=15 (BCG-unvaccinated infants at 1 year time point). Statistics for
567 comparisons between vaccinated and unvaccinated infants are Mann-Whitney U tests. *
568 indicates $p<0.05$.
569
570 **FIGURE 3. Increased capacity to inhibit the *in vitro* growth of mycobacteria in PBMC**
571 **from BCG-vaccinated infants that correlates with polyfunctional T-cell response.**
572 Cryopreserved PBMC prepared from venous blood samples obtained 4 months and 1 year
573 post-BCG from vaccinated infants or from age-matched, unvaccinated infants were recovered
574 and incubated with BCG for 4 days after which remaining mycobacteria were quantified by
575 BACTEC MGIT tubes. Inhibition of BCG growth is indicated by the log CFU of remaining
576 mycobacteria after incubation with PBMC obtained at the 4 month (A) or 12 month (B) time
577 point from BCG-vaccinated (+) or BCG-unvaccinated (-) infants. Sample numbers are n=8
578 (BCG-vaccinated infants at 4 months post-BCG); n=8 (BCG-vaccinated infants at 1 year
579 post-BCG); n=7 (BCG-unvaccinated infants at 4 month time point) and n=7 (BCG-
580 unvaccinated infants at 1 year time point). For all infant samples where growth inhibition and
581 ICS data was available (n=12), scatter plots of log CFU versus frequency of polyfunctional
582 CD4+ T-cells (C) or versus IL-17+CD4+ T-cells (D) were drawn. Solid symbols indicate 4-
583 month samples; open symbols indicate 12-month samples; circles indicate samples from
584 vaccinated infants; triangles indicate samples from unvaccinated infants. The Mann-Whitney
585 U test was used to compare growth inhibition between vaccinated and un-vaccinated groups.
586 Spearman's rank correlation coefficient was calculated between growth inhibition and ICS
587 frequencies and significant correlations ($p<0.05$) are indicated (*).

Figure 1

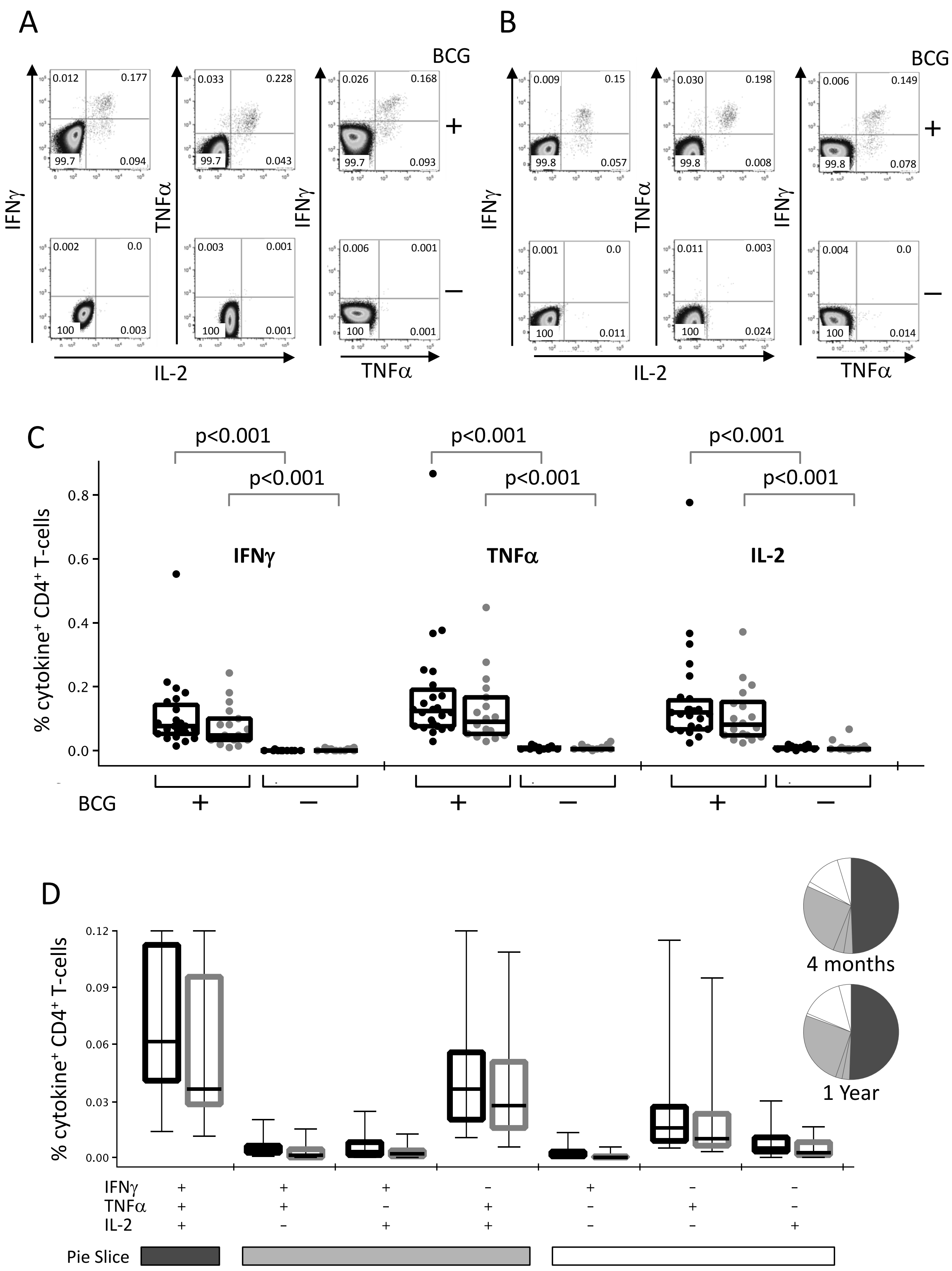

Figure 2

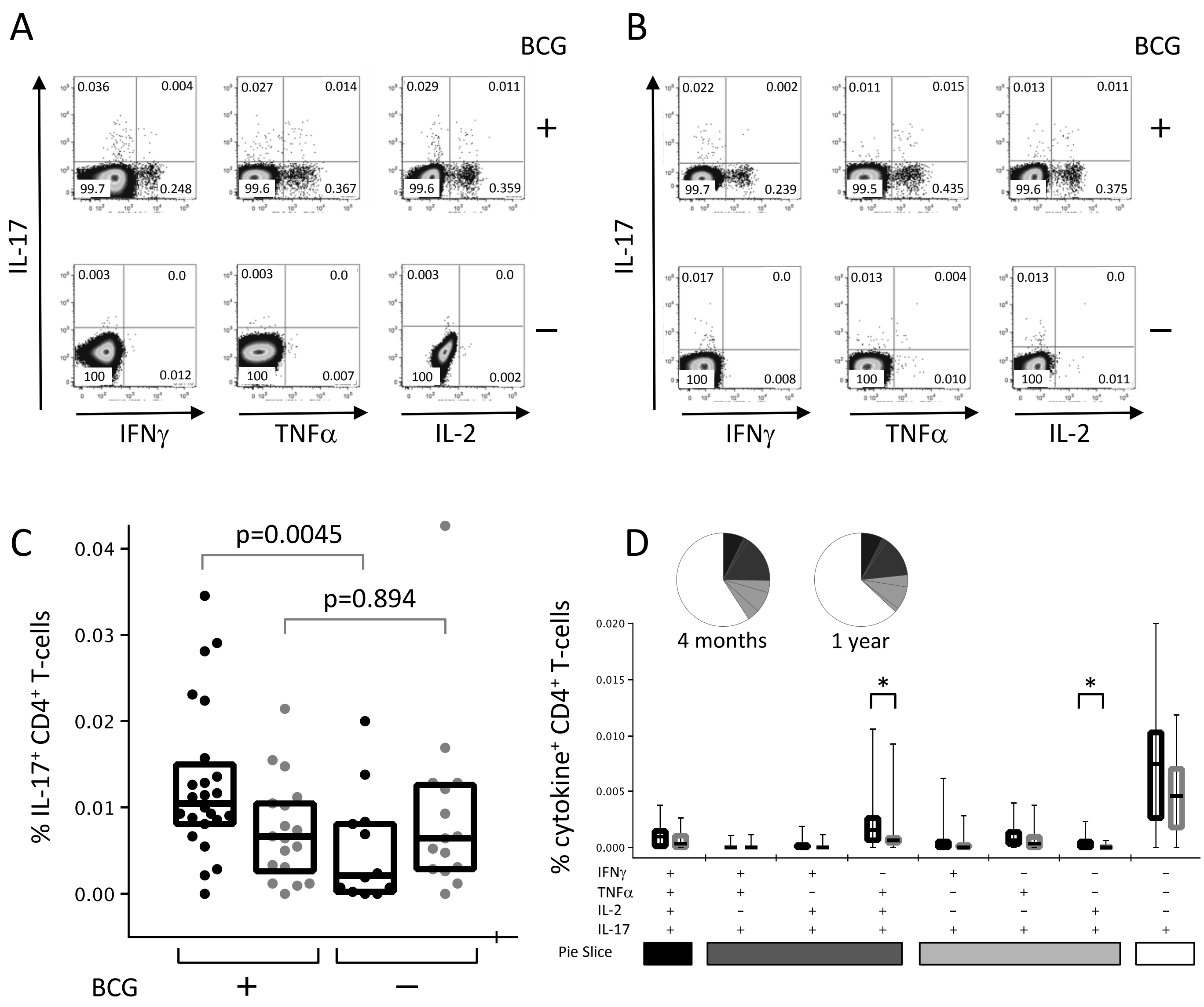

Figure 3