Unique T cell signatures are associated with reduced *Chlamydia trachomatis* reinfection in a highly exposed cohort

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ABSTRACT

Chlamydia trachomatis (CT) is the most common bacterial sexually transmitted infection (STI) in the United States, despite effective antibiotics. Information regarding natural immunity to CT will inform vaccine design. The objectives of this study were to determine immune cell populations and functional features associated with reduced risk of CT reinfection or endometrial CT infection. PBMCs were collected from a cohort of CT-exposed women who were tested for CT and other STIs at the cervix and endometrium (to determine ascension) and were repeatedly tested over the course of a year (to determine reinfection). Mass cytometry identified major immune populations and T cell subsets. Women with CT had increased CD4+ effector memory T cells (TEM) compared to uninfected women. Specifically, Th2, Th17, and Th17 DN CD4+ TEM were increased. Th17 and Th17 DN CD4+ central memory T cells (TCM) were increased in women who did not experience follow-up CT infection, suggesting that these cells may be important for protection. These data indicate that peripheral T cells display distinct features that correlate with natural immunity to CT and suggest that the highly plastic Th17 lineage plays a role in protection against reinfection.

37 **INTRODUCTION**

38

39 *Chlamydia trachomatis* (CT) is the most common bacterial sexually transmitted infection
40 (STI) globally (1). In women, CT infects the cervix and can ascend to the uterus and oviducts to
41 cause pelvic inflammatory disease that can result in pelvic pain, infertility, and ectopic pregnancy
42 (2). Although antibiotic treatment is effective, 80% of infections are asymptomatic and may go
43 undetected (3), contributing to the risk for chronic sequelae (4).

44 Although repeated CT infections are common, they elicit partially protective immunity.
45 Women who spontaneously cleared infection without treatment were less likely to become
46 reinfected (5) and persons with confirmed prior infection have lower chlamydial loads (6).
47 Determining the aspects of natural immunity that drive protection against CT will advance
48 understanding of chlamydial immunity and aid design of an effective vaccine. Severe combined
49 immune deficient (SCID), RAG-1-/-, TCRα-/-, CD4-/-, and MHCII-/- mice display delayed or failed
50 resolution of *Chlamydia* infection, while MHCI-deficient (B2M-/-), B cell-deficient (μMT), and CD8-
51 /- mice resolve infection with comparable kinetics to wild-type (7-11). These data emphasize the
52 importance of CD4 T cell mediated immunity in a murine model of severe genital tract disease.

53 The T Cell Response Against Chlamydia (TRAC) cohort, a unique resource containing
54 clinical samples and information from women highly exposed to CT and other STIs, was
55 established to improve understanding of human natural immunity to CT (12). We observed that
56 CD4 T cells producing CT-specific IFNγ were associated with protection from reinfection (13),
57 consistent with findings of others (14), and we additionally determined that CD8 T cells producing
58 CT-specific IFNγ were associated with protection from infections that ascended to the
59 endometrium (13). Further, we identified a single nucleotide polymorphism in the gene for CD151,
60 a tetrospanin molecule important for immune cell adhesion and migration and T cell proliferation,
61 as a candidate genetic marker for reduced susceptibility for endometrial CT infection (15).
62 Examining the chlamydial-specific responses of TRAC participants, we determined that anti-CT

63 antibodies are insufficient to protect women from ascending or recurrent infections (16, 17). TRAC
64 was specifically designed to characterize two important clinical outcomes of CT infection: (1)
65 distinguishing lower tract infection from ascended upper tract infection, which has direct
66 implications for disease severity, and (2) rates of reinfection, determined by diagnostic STI testing
67 and self-reports at 1-, 4-, 8-, and 12-months post-enrollment. In summary, these data indicate that
68 CD4 T cells, and to a lesser extent CD8 T cells, but not antibody, contribute to protection against
69 CT reinfection or ascension. The objective of this study was to further probe specific, cell-
70 mediated features of natural protective chlamydial immunity by using mass cytometry to
71 immunophenotype peripheral blood from TRAC participants for whom reinfection and ascension
72 status were defined.

73 Mass cytometry enabled inclusion of sufficient markers to determine broad populations of
74 immune cells while simultaneously focusing on CD4 and CD8 T cell subsets with distinct
75 functions, such as T follicular helper (Tfh) cells, regulatory T (Treg) cells, central memory T (TCM)
76 cells, effector memory T (TEM) cells, and stem-cell like memory T (TSCM) cells. TEM and TCM
77 T-helper cell subsets were defined by surface expression of chemokine receptors CCR6, CXCR3,
78 and CCR4 (18-20). Th1 cells (CCR6-CXCR3+CCR4-) produce mainly IFNγ and are important for
79 macrophage activation, phagocytosis, and bacterial defense, while Th2 cells (CCR6-CXCR3-
80 CCR4+) produce mainly IL-4, IL-5, and IL-13 and are important for supporting antibody production
81 (21). Th17 cells are a highly plastic family. Classical Th17 cells (CCR6+CXCR3-CCR4+) produce
82 primarily IL-17 and are important for neutrophil recruitment and defense at mucosal sites (22);
83 Th1/17 cells (CCR6+CXCR3+CCR4-) share Th1 and Th17 functionality, respond to IL-12 and IL-
84 23, and produce mostly IFNγ (23); Th17 double positive (DP) cells (CCR6+CXCR3+CCR4+)
85 produce IFNγ and low levels of IL-17 (19), and double negative (DN) cells (CCR6+CXCR3-CCR4-
86) preferentially produce IL-17 and express transcriptional markers of early Th17 development (IL-
87 17F, STAT3), lymph-node homing (CCR7, CD62L), follicular help (CXCR5, BCL6, ASCL2), and
88 self-renewal (LEFI, MYC, TERC) (19, 24). We observed increased frequencies of CD4+ memory

89 Th17 and Th17 DN cells in participants with active CT infection and determined that these cells
90 were additionally enriched in participants protected from recurrent infection during the year they
91 were enrolled in the study.
92
93 **RESULTS**
94
95 **Overall participant characteristics.**
96 This study acquired CyTOF data from PBMCs of 82 of the 246 participants enrolled in
97 TRAC (12). The sociodemographic characteristics and self-reported STI diagnoses, and exposure
98 histories of this sub-cohort resemble those previously described for the full cohort (Supplemental
99 Table 1, Supplemental Table 2) (12).
100
101 **CT genital tract infection elicits monocytes, plasma cells, and CD4 T effector memory cells**
102 **in the periphery.**
103 Study participants were classified by their infection status at enrollment: Uninfected (CT-
104 ;GC/MG-), CT monoinfection (CT+;GC/MG-), or CT coinfection with *Neisseria gonorrheoae* (GC)
105 and/or *Mycoplasma genitalium* (MG) (CT+;GC/MG+), and their PBMCs, collected at enrollment
106 and 1 month post treatment (1M), were immunophenotyped by CyTOF using a 33-marker panel
107 (Fig 1A, Supplemental Table 3). The manual gating hierarchy and definitions used to determine
108 frequencies of each cell type are outlined in Supplemental Table 4. Frequencies of total myeloid
109 cells were increased in CT+ women at enrollment and remained elevated at 1M, regardless of
110 coinfection (Fig 1B). Frequencies of total B cells, CD4 T cells, and CD8 T cells were similar across
111 all groups (Fig 1C-E). While dendritic cells (myeloid and plasmacytoid) and NK cell frequencies
112 were similar across patient subgroups (data not shown), classical, intermediate, and non-classical
113 monocytes were increased in CT+ women at enrollment and 1M, regardless of coinfection with
114 significant increases noted in non-classical monocytes (Fig 1F-H). Interestingly, frequencies of

115 plasma cells were increased in CT+;GC/MG- participants, but not CT+;GC/MG+ participants, at
116 enrollment and 1M (Fig 1I). Previous studies have shown that CT drives a strong and specific
117 antibody response (12, 25), which would be supported by a high frequency of plasma cells.
118 Frequencies of CD4 TEM cells were significantly increased in CT+ women at enrollment and 1M,
119 regardless of coinfection (Fig 1J). CD8 TEM were slightly increased in CT+ women (Fig 1K),
120 although to a lesser extent than CD4 TEM. In contrast, CD4 and CD8 TCM frequencies were
121 similar or slightly reduced in CT+ women compared to uninfected (Fig 1L, M). Frequencies for
122 populations defined in Fig 1A but not shown individually were not different between groups (data
123 not shown). Overall, these data demonstrate that CT infection drives increased frequencies of
124 peripheral monocytes, plasma cells, and CD4 effector memory cells, responses that remain
125 elevated for at least one month after treatment with antibiotics.
126
127 **Participants with CT have increased peripheral frequencies of long-lived Th2 and Th17-**
128 **lineage CD4 TEM cells compared to uninfected participants.**
129 It is broadly accepted that CD4 TEM, and specifically IFNγ-producing Th1 cells, are the
130 primary contributors to protection against CT in animals and humans (13, 26). Therefore, the
131 CyTOF antibody panel was designed with an investigational focus on T-cell subsets. Initial
132 investigation of major cell populations revealed a significant increase in peripheral CD4 TEM cells
133 in participants with CT (Fig 1J). Expression of the chemokine receptors CCR6, CXCR3, and CCR4
134 further characterized CD4 and CD8 T-helper subtypes of peripheral TEM (Fig 2A) (18-20).
135 Frequencies of CD4 TEM Th1 cells were not different between CT+ and uninfected individuals
136 (Fig 2B). Frequencies of CD4 TEM Th2 cells were markedly increased in CT+ participants at
137 enrollment and 1M, regardless of coinfection (Fig 2C), which is consistent with strong antibody
138 responses detected in response to CT (12, 25). Frequencies of CD4 TEM Th17 and Th17 DN
139 cells were increased in CT+;CT/MG- participants at enrollment and 1M, whereas there was no

140 difference in frequencies of Th1/17 or Th17 DP cells (Fig 2D-G). These differences were
141 maintained in CT+;GC/MG+ participants for CD4 TEM Th2 but not Th17 lineage cells (Fig 2C-F).
142 We similarly applied chemokine receptor definitions to investigate cells comprising the
143 CD8 memory compartment (Tc1, Tc2, Tc17, etc.). Frequencies of CD8 TEM Tc1 and Tc2 were
144 significantly increased in CT+;GC/MG- participants compared to uninfected (Fig 2H-I). There was
145 no change in CD8 TEM Tc1/17 (Fig 2J), but Tc17 DN cells trended toward an increase in
146 CT+;GC/MG- participants (Fig 2K), both of which parallel the results for CD4 TEM subsets
147 described above. CD8 TEM Tc17 and Tc17 DP were not detected. There were no differences in
148 either CD4 or CD8 TCM subtypes (data not shown), which is not surprising considering that
149 overall frequencies of CD4 and CD8 TCM were unchanged (Fig 1L-M). Frequencies of Treg and
150 Tfh cells were also determined but were not different between uninfected and CT+ participants
151 (data not shown). Overall, these results suggest that CT infection drives a peripheral adaptive
152 immune response dominated by CD4 and CD8 TEM Th2/Tc2 and CD4 TEM Th17-lineage cells.
153
154 **Frequencies of CD4 TSCM are enriched in the peripheral blood of participants with**
155 **endometrial CT infection.**
156 All participants were tested for CT, GC, and MG infection at cervical and endometrial sites
157 at enrollment, enabling bisection of the CT+;GC/MG- and CT+;GC/MG+ groups into CT infections
158 that had ascended to the upper genital tract (Endo+) and CT infections that were limited to the
159 cervix (Endo-). All major populations and T cell subsets were defined as above (Supplemental
160 Table 4), and comparisons of population frequencies performed between Endo+ and Endo-
161 participants. Women who were CT+;GC/MG- and Endo+ exhibited significantly increased
162 frequencies of CD4 TSCM, which was not observed in CT+;GC/MG+ women (Fig 3A). In
163 CT+;GC/MG- and Endo- participants, CD8 TEM and TCM Tc17 DN cells trended toward increase
164 (Fig 3C-D), suggesting expansion of these cells may contribute to protection from ascending CT

165 infection. Overall, however, there were comparatively minimal differences in peripheral blood T
166 cell populations between Endo+ and Endo- CT.
167
168 **CD4 TCM Th1 and Th17 family subsets are enriched in peripheral blood of women without**
169 **documented CT reinfection.**
170 Individuals treated for CT often experience repeat infections (6). Nevertheless, evidence
171 for acquired natural immunity includes documented increased resistance to CT reinfection in
172 women who previously spontaneously cleared an infection (14), and decreased CT infection rates
173 with increasing age after controlling for sexual activity (27). The TRAC study included a
174 longitudinal component designed to investigate attributes of the immune response contributing to
175 resistance to reinfection. Participants completed follow-up visits at 1, 4, 8, and 12 months where
176 they underwent diagnostic testing (cervix) and were asked to report any STI diagnoses that
177 occurred since their last visit. Using this information, we again bisected CT+;GC/MG- and
178 CT+;GC/MG+ groups into FU+ (positive for CT by diagnostic test or self-report at any follow-up
179 visit) and FU- (completed at least 3 out of 4 follow-up visits, and negative by diagnostic test and
180 self-report at all visits). Nine participants completed fewer than 3 follow-up visits and were
181 excluded from this analysis.
182 We compared frequencies of manually gated T cell populations between FU- and FU+
183 individuals. All cell populations described above (Supplemental Table 4) were defined but only
184 significant findings are presented in Figure 4. Frequencies of total CD4 and total CD8 T cells and
185 frequencies of CD4 and CD8 stem-cell like memory (TSCM) cells were enriched in FU-
186 participants without co-infection at enrollment and remained expanded 1 month post treatment
187 (Fig 4A-D). CD4 Treg cells were enriched in FU- participants at the enrollment visit (Fig 4E).
188 Interestingly, frequencies of CD4 TEM Th2 cells were increased in FU+ participants (Fig 4F),
189 suggesting that although these cells are induced by CT infection, they are not protective. In
190 contrast, frequencies of CD4 TCM Th1, Th17, Th1/17, and Th17 DN cells were increased in FU-

191 participants (Fig 4G-J). These data suggest that T cells, specifically CD4 and CD8 TSCM and
192 CD4 TCM Th1/Th17 lineage cells are important for developing natural immunity against incident
193 CT infection. Interestingly, differences in frequencies of T cell subsets between FU- and FU+
194 participants were only detected in CT+;GC/MG- and not CT+;GC/MG+ participants, which
195 suggests that immune responses specific to CT are diluted by coinfection.
196

197 **DISCUSSION**

198 Using samples from a uniquely characterized and clinically-relevant cohort, this study
199 immunophenotyped responses to CT infection with high dimensional analysis of surface marker
200 expression on PBMCs and found specific T cell populations correlated with reduced incidence of
201 CT reinfection. Overall, broad immune cell populations in peripheral blood were different between
202 uninfected and CT+ participants, but similar between CT monoinfection and coinfection. Notably,
203 CT+ participants had increased frequencies of mononuclear myeloid cells, consistent with an
204 inflammatory response to an acute infection. CT+ participants displayed increases in frequencies
205 of non-classical (CD14-CD16+) monocytes, important for antigen presentation, T cell proliferation,
206 patrolling tissues for signs of injury, and increased Fc receptor-mediated phagocytosis (28).
207 Frequencies of monocytes remained elevated in some samples of PBMCs collected one month
208 later from participants who were CT+ at enrollment, likely driven by ongoing inflammation induced
209 by danger associated molecular patterns released from bacteria and dying cells.

210 While frequencies of immune populations were largely similar in CT+ participants
211 regardless of coinfection, plasma cells were elevated in participants with CT monoinfection, but
212 their frequencies in CT+;GC/MG+ participants were similar to uninfected. This suggests that
213 plasma cells are driven by CT infection, consistent with well-documented robust but non-
214 protective antibody responses to CT infection (16, 29, 30), but that this response is suppressed
215 or impeded by coinfection with other STIs. *N. gonorrhoeae* opacity proteins bind
216 carcinoembryonic antigen-related cellular adhesion molecule 1 (CEACAM1 or CD66a) on B cells,

217 leading to inhibitory signals which promote B cell death (31). Plasma cells also express CD66a
218 (32). Thus, in women coinfected with GC, opacity protein interactions with CD66a could contribute
219 to reduced numbers of plasma cells, especially in women with chronic GC infection.

220 CD4 TEM frequencies were strikingly increased in CT+ participants compared to CD8
221 TEM frequencies. These data are consistent with our prior investigation of CT antigen specific
222 responses in a subset of TRAC participants where we observed higher levels of IFNγ production
223 and a greater breadth of recognition of CT antigens among CD4 T cells compared to CD8 T cells
224 (13). That CD8 TEM frequencies were not increased is also consistent with engaging the PD-1
225 receptor on CD8 T cells by antigen presenting cells inhibits generation of CT-specific memory
226 CD8 T cells during murine chlamydial infection (33). Frequencies of TEM but not TCM were
227 increased in CT+ compared to uninfected participants in our study. In contrast, an analysis of *C.*
228 *pneumoniae*-specific peripheral blood T cells in healthy donors found that CD4 TCM were
229 enriched compared to TEM (34). These contrasting data may be explained by the difference
230 between acute infection experienced by TRAC participants and relatively remote infection
231 experienced by individuals in the *C. pneumoniae* study.

232 Further investigation of CD4 TEM subsets revealed that CT+ participants displayed higher
233 frequencies of peripheral Th2, Th17, and Th17 DN cells, with unchanged frequencies of Th1 cells.
234 Th1, Th2, and Th17 cells support different aspects of the overall downstream immune response.
235 CD4 Th1 cells are classically defined by production of IFNγ and expression of CXCR3 and/or
236 CCR5 (18). They are well-documented contributors to clearance of *Chlamydia* and protection from
237 reinfection in mouse models (35-37) and associate with resistance to CT reinfection in humans
238 (13, 14). We did not find evidence for expansion of Th1 cells in the peripheral blood of CT+
239 participants compared to uninfected. The absence of such an important protective cell type in the
240 periphery may explain why CT reinfection is so common. However, Th1 cells express the
241 chemokine receptors CXCR3 and CCR5, which bind the chemokines CXCL10 and CCL5
242 (RANTES), respectively (38). We previously reported detecting both of these chemokines in

243 cervical secretions of TRAC participants (39), and they are highly expressed in genital tract
244 tissues of *C. muridarum* infected mice (37). Thus, it is possible that despite overall expansion of
245 Th1 cells, they were preferentially recruited to the site of infection and consequently not increased
246 in the periphery. In contrast, peripheral CD4 Th2 cells were significantly enriched in CT+ women,
247 consistent with the fact that Th2 responses are generally supportive of antibody production and
248 that CT infection is known to drive strong but non-protective antibody responses (6, 12, 16).

249 Th17 cells are a highly plastic family of T-helper cells. They are mainly defined by their
250 expression of CCR6 (40) but can gain or lose chemokine receptors and their associated effector
251 functions (e.g., cytokine production) (41). Th17 cells can be pathogenic, but they are important
252 contributors to protection against mucosal pathogens such as *Mycobacterium tuberculosis* and
253 *Bordetella pertussis* (42, 43). We used expression of CCR6, CXCR3, and CCR4 to define four
254 subsets of Th17 family cells: classical Th17, Th1/17, Th17 DP, and Th17 DN (19). Th17 DN cells
255 express transcriptional markers of early Th17 development (IL-17F, STAT3), lymph-node homing
256 (CCR7, CD62L), follicular help (CXCR5, BCL6, ASCL2), and self-renewal (LEFI, MYC, TERC)
257 (19), consistent with their highly plastic nature. We found increased frequencies of classical Th17
258 and Th17 DN cells in CT+ participants compared to uninfected. These findings, when considered
259 alongside classifications of whether participants experienced CT infection that ascended to the
260 endometrium (Endo+ vs Endo-) and whether CT reinfection was documented over the course of
261 one year of follow-up visits (FU+ vs FU-), provided important insights into specific responses that
262 may be protective. Future studies will examine Th17 family subsets to determine the functional
263 cytokine responses of Th17 and Th17 DN cells in cervical and endometrial tissues from CT-
264 infected women.

265 Peripheral immune cell populations were similar overall between participants with
266 endometrial CT infections and those that had CT infections limited to the cervix. Frequencies of
267 CD4 TSCM were significantly increased in Endo+ and FU- participants. This is consistent with our
268 prior finding that endometrial infection was associated with reduced risk of follow-up CT infection

269 (12), likely because the increased bacterial burden associated with endometrial infection drives a
270 more robust memory response. CD8 TEM Tc17 DN cells trended toward increased frequencies
271 in CT+;GC/MG- Endo- participants, consistent with our previous study showing that CT-specific
272 CD8 T cells weakly associated with reduced CT ascension (13). However, evaluation of immune
273 cell populations in the genital tract is necessary to define the specific features of immunity that
274 prevent or limit ascending infection. Future studies will investigate immune cells from cervical and
275 endometrial TRAC samples.

276 In contrast to the relatively minor differences identified between Endo+ and Endo-
277 participants, several T cell subtypes were found to be strongly associated with presence or
278 absence of recurrent CT infection. Our detection of increased frequencies of total CD4 T cells in
279 FU- participants at enrollment and maintenance of an increase one month post treatment is
280 indicative of developing natural immunity in some participants. We also detected increased
281 frequencies of CD4 and CD8 TSCM in FU- individuals. TSCM resemble naïve T cells in their
282 expression of CD45RA and CCR7, but they also express CD95, a marker of antigen experience
283 and memory (44). They are minimally differentiated memory cells that have a high propensity for
284 stemness and self-renewal as well as the multipotency to differentiate into a broad range of
285 memory T-helper phenotypes (44), which may explain why they are associated with FU-
286 outcomes, The importance of the quality of the T cell response is demonstrated by the contrasting
287 subsets that were expanded in FU- and FU+ participants. Expansion of TEM Th2 cells, which
288 were strikingly increased overall in CT+ participants, was associated with a FU+ outcome, while
289 frequencies of TCM Th1 cells, which were not different between CT+ and uninfected participants,
290 were associated with FU- status. An important role for Th17-lineage cells in protection from
291 reinfection is suggested by the striking increase in TCM Th17, along with increased frequencies
292 of TCM Th1/17 and TCM Th17 DN cells in FU- participants, again suggesting that Th17-family
293 cells may contribute to CT natural immunity.

294 The finding that Th1 and Th17 subsets were associated with reduced reinfection (and that
295 Th2 responses were not) provides the opportunity to design vaccines that induce more protective
296 responses than natural immunity. Different vaccine platforms and adjuvants can shape different
297 T-helper responses (45). CAF01, a Th1/Th17 adjuvant, combined with recombinant chlamydial
298 antigen elicited resident memory Th1/Th17 responses that increased protection of mice against
299 chlamydial challenge (46). Together, these findings support the design of vaccines that prioritize
300 CD4 Th1 and Th17-polarized responses to elicit the best protection against CT.

301 Our study represents a major advancement in the understanding of natural immunity to
302 CT in humans, but it is somewhat limited because we defined populations increased in CT+
303 participants compared to uninfected participants, but the cells were not restimulated with CT
304 antigens to confirm their CT-specificity. Immunophenotyping PBMCs directly without restimulation
305 allowed us to confidently define T cell subsets based on chemokine receptors without the risk of
306 downregulation upon restimulation. However, the important open question of whether Th17 and
307 Th17 DN cells produce CT-specific IL-17 and/or IFNγ remains to be addressed in future studies.
308 Another limitation of this study is that cell population frequencies could not be correlated with
309 endometrial inflammation and pelvic inflammatory disease. Many of the women participating in
310 TRAC selected contraceptive methods that promote thin uterine lining, making it difficult to acquire
311 sufficient tissue for evaluation of pathology. However, ongoing studies are investigating local T
312 cell responses in endometrial tissue sections from participants with sufficient tissue.

313 Overall, we found that frequencies of CD4 TEM, specifically Th2, Th17, and Th17 DN
314 subsets, were enriched in individuals with active CT infection. We also identified that increased
315 frequencies of CD4 TCM Th1, Th17, and Th17 were associated with individuals who had no
316 documented reinfection over the course of a year. Together, these findings increase our
317 understanding of natural immunity to CT and inform strategies for future vaccine design.

318

319 **METHODS**

320

321 **Patient Populations.**

322 A subset of a previously described T Cell Response Against Chlamydia (TRAC) cohort (12) was
323 used for this study.

324

325 **Definition of Clinical Classifications**

326 *Uninfected, CT+;GC/MG-, and CT+;GC/MG+ Groups:*

327 Nucleic acid amplification testing (NAAT) was used to test cervical swabs collected from study
328 particpants at enrollment for CT, *Neisseria gonorrhoeae* (gonococcus, GC), and *Mycoplasma*
329 *genitalium* (MG). Particpants whose swabs tested negatively for all 3 STIs at enrollment were
330 classified as “uninfected”. Participants testing positively for CT but negative for GC and MG were
331 classified as “CT+;GC/MG-”. Enrollees testing positively for CT and GC, MG, or both were
332 classified as “CT+;GC/MG+”. Of 82 total TRAC participants studied, 12 were uninfected, 44 were
333 CT+;GC/MG-, and 26 were CT+;GC/MG+ (Supplemental Table 5).

334

335 *Endometrial positive and endometrial negative CT:*

336 Endometrial biopsies were also NAAT tested by to determine whether infection was limited to the
337 cervix or had ascended to the upper genital tract. Within CT+;GC/MG- and CT+;GC/MG+ groups,
338 enrollees testing positively for CT at the cervix but not the endometrial biopsy were classified as
339 Endo-, while those testing positively for CT at both sites were classified as Endo+. No participants
340 tested positive for CT solely in the endometrial sample. Of 44 CT+;GC/MG- participants, 19 were
341 Endo+ and 25 were Endo-. Of 26 CT+;GC/MG+ participants, 11 were Endo+ and 15 were Endo-
342 (Supplemental Table 5).

343

344 *Follow-up CT positive and follow-up CT negative:*

345 NAAT testing was performed on cervical brush samples at enrollment and at 1, 4, 8, and 12 month
346 follow-up visits. Participants with a positive cervical CT test at enrollment and a positive cervical
347 CT test or self-reported CT diagnosis from an outside clinic in the time since last visit at any follow-
348 up visit (regardless of how many visits they completed) were classified as follow-up positive (FU+).
349 Participants that tested positive for cervical CT at enrollment, completed at least 3 out of 4 follow-
350 up visits, tested negative for cervical CT at all follow-up visits, and self-reported no CT diagnosis
351 from an outside clinic in the time since last visit at any follow-up visit were classified as follow-up
352 negative (FU-). 9 participants attended less than 3 follow-up visits (FU N/A) and were excluded
353 from follow-up analysis. Of CT+;GC/MG- participants, 19 were FU-, 21 were FU+, and 4 were FU
354 N/A. Of CT+;GC/MG+ pariticpants, 9 were FU-, 12 were FU+, and 5 were FU N/A (Supplemental
355 Table 5). All uninfected samples were FU- and the group was therefore not bisected.
356
357 **PBMC Processing**
358 Whole blood samples from individual patients at each visit were diluted with equal volume of PBS
359 and peripheral blood mononuclear cells (PBMCs) were isolated via a density gradient using
360 lymphocyte separation media (Corning). Cells were frozen in 90% FBS with 10% DMSO and
361 stored in liquid nitrogen until thawed for CyTOF analysis.
362
363 **Mass Cytometry**
364 Mass cytometry (CyTOF) was performed on 156 total PBMC samples (Supplemental Table 5). A
365 mass cytometry panel comprising of 33 surface markers (Supplemental Table 3) was used to
366 profile immune cell populations in detail. Frozen PBMCs were thawed in a 37°C water bath, and
367 an equal amount of pre-warmed complete RPMI (cRPMI) with 50 units/mL benzonase (Sigma)
368 was added dropwise. Cells were then centrifuged at 300*xg* for 5 min at room temperature (RT).
369 Pellets were washed in cRPMI and enumerated using trypan blue and a hemocytometer. 3 x 10^6
370 viable participant cells and 1 x 10^6 PBMCs from a common negative control donor were

371 transferred to one FACS tube and washed with PBS (CyTOF grade, Rockland Immunochemicals
372 Inc.). Dead cells were stained with 1 μM Cell-ID Cisplatin 198-Pt (Fluidigm) for 5 min at RT and
373 washed twice with cell staining buffer (CSB, Fluidigm). An antibody cocktail comprising 33
374 antibodies (Supplemental Table 3) and human Fc block (eBioscience) in was added to cells in 50
375 μL staining volume and incubated for 30 min at RT. Cells were washed twice with CSB followed
376 by secondary antibody (Anti FITC-144Nd) incubation. Stained cells are fixed with 2% PFA in PBS
377 for 1 hr at RT followed by two washes in PBS. 1 mL of 1:2000 Cell-ID Intercalator-Ir-125 μM
378 (Fluidigm) solution in fix and perm buffer (Fluidigm) was added to the cells to stain the DNA and
379 incubated for 30 min at RT or 4°C overnight. Cells were further washed with CSB twice and
380 resuspended in CAS (Cell Acquisition Solution; Fluidigm). Cell concentrations were adjusted to
381 0.5 x 10^6 cells/mL in CAS with Four Element Calibration beads (Fluidigm). 200,000 cells per
382 sample were acquired on a Helios instrument (Fluidigm) at a flow rate of 250 cells/sec by the UNC
383 Mass Cytometry Core.
384
385 **Data Analysis**
386 *Data Cleaning.* Raw CyTOF files were cleaned using the Pathsetter algorithm (47). All samples
387 were of good quality and viability. FCS files containing cleaned viable events were created and
388 used for remaining analysis.
389 *Manual Gating Phenotypic Analysis.* All analysis was done in Cytobank (Beckman Coulter) and
390 FlowJo (BD) software. The manual gating strategy and surface marker designations selected for
391 immune cell phenotypes are summarized in Supplemental Table 4.
392
393 **Statistics**
394 Statistical calculations were all computed in R. For comparisons between uninfected,
395 CT+;GC/MG-, and CT+;GC/MG+, a non-parametric Kruskal-Wallis test followed by Dunn's test
396 and Bonferroni correction for multiple comparisons was used. For comparisons between Endo-

397 vs Endo+ and FU- vs FU+, a non-parametric Wilcoxon ranked sum test was used. $P<0.05$ was
398 used to determine a significant result. Significance symbols used in figures are defined as:
399 *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$.
400
401 **Study Approval**
402 The institutional review boards for human research of the University of Pittsburgh and the
403 University of North Carolina approved the study protocol. All participants provided written informed
404 consent at the time of enrollment and agreed to be contacted to return for follow-up visits 1, 4, 8,
405 and 12 months after enrollment.
406
407 **AUTHOR CONTRIBUTIONS**
408 KSY, AK, XZ, and TD designed research studies. KSY and AK conducted experiments. HCW and
409 SLH collected participant samples and performed diagnostic testing. KSY, CL, and BB analyzed
410 data. KSY, CMO and TD wrote the manuscript.
411
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413
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421

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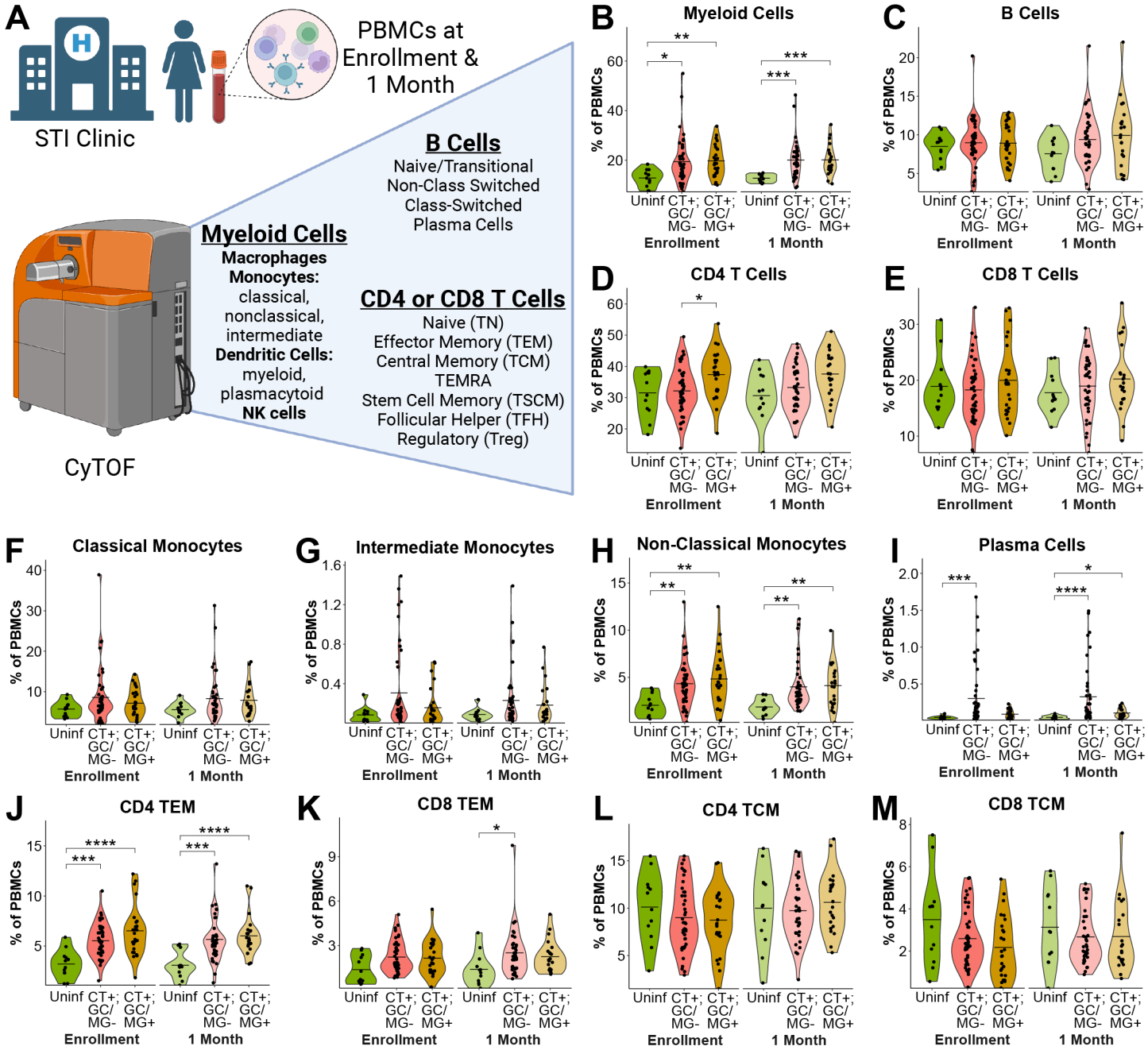

Figure 1. CT genital tract infection elicits monocytes, plasma cells, and CD4 T effector memory cells in the periphery. (A) Major immune populations defined by manual gating in peripheral blood of 82 participants at enrollment and 1 month post-treatment. (B-M) Population percentages of total PBMCs. Horizontal bar represents mean. Significance was determined by Kruskal-Wallis test followed by Dunn's test and Bonferroni correction for multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

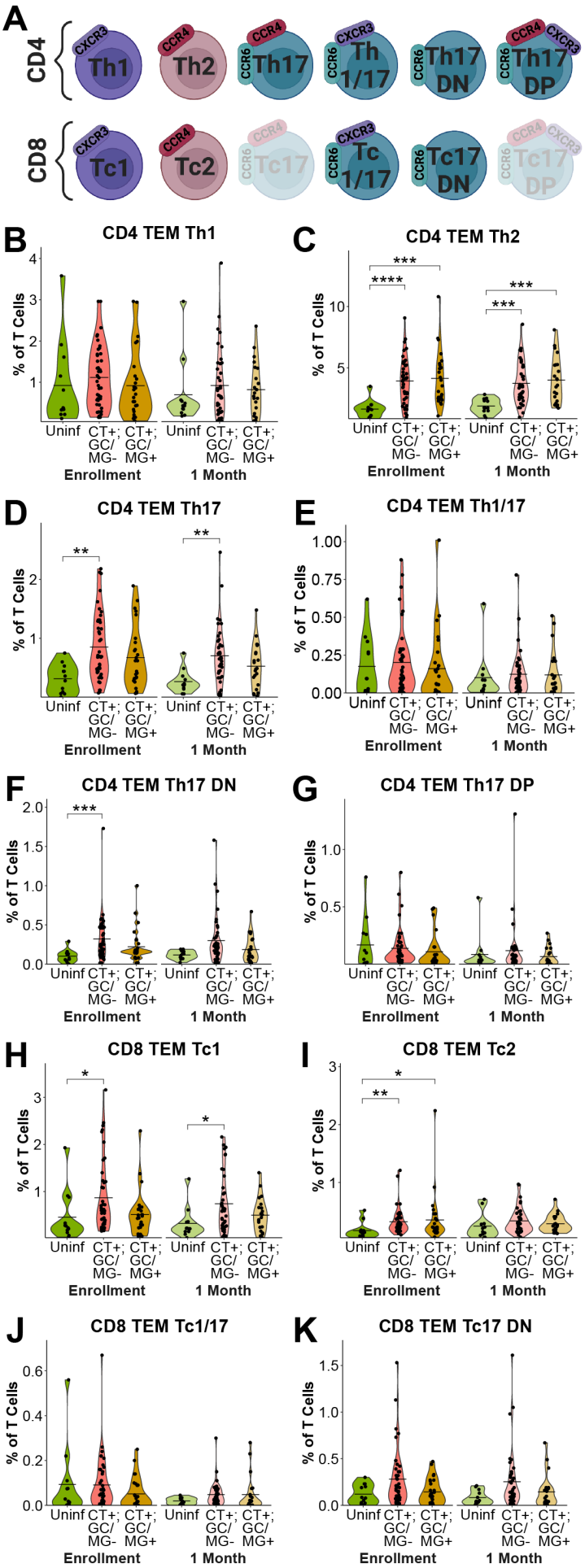

Figure 2. Participants with CT have increased peripheral frequencies of long-lived Th2 and Th17-lineage CD4 TEM cells compared to uninfected participants.

(A) Subsets of CD4 and CD8 TEM were identified by expression of chemokine receptors CCR6, CXCR3, and CCR4. (B-K) Population percentages of total T Cells. Horizontal bar represents mean. Significance was determined by Kruskal-Wallis test followed by Dunn's test and Bonferroni correction for multiple comparisons. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

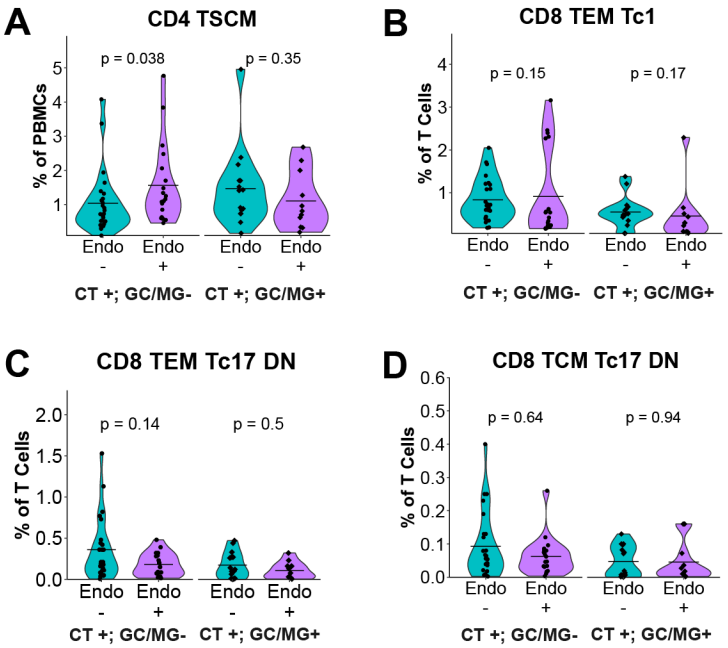

Figure 3. Frequencies of CD4 TSCM are enriched in the periphery of participants with endometrial CT infection.

Frequencies of manually gated T cell subpopulations were stratified by cervix only CT (Endo-) or cervix and endometrial CT (Endo+) status. (A-D) T cell subpopulation percentages. Horizontal bar represents mean. Significance was determined by Wilcoxon rank-sum test.

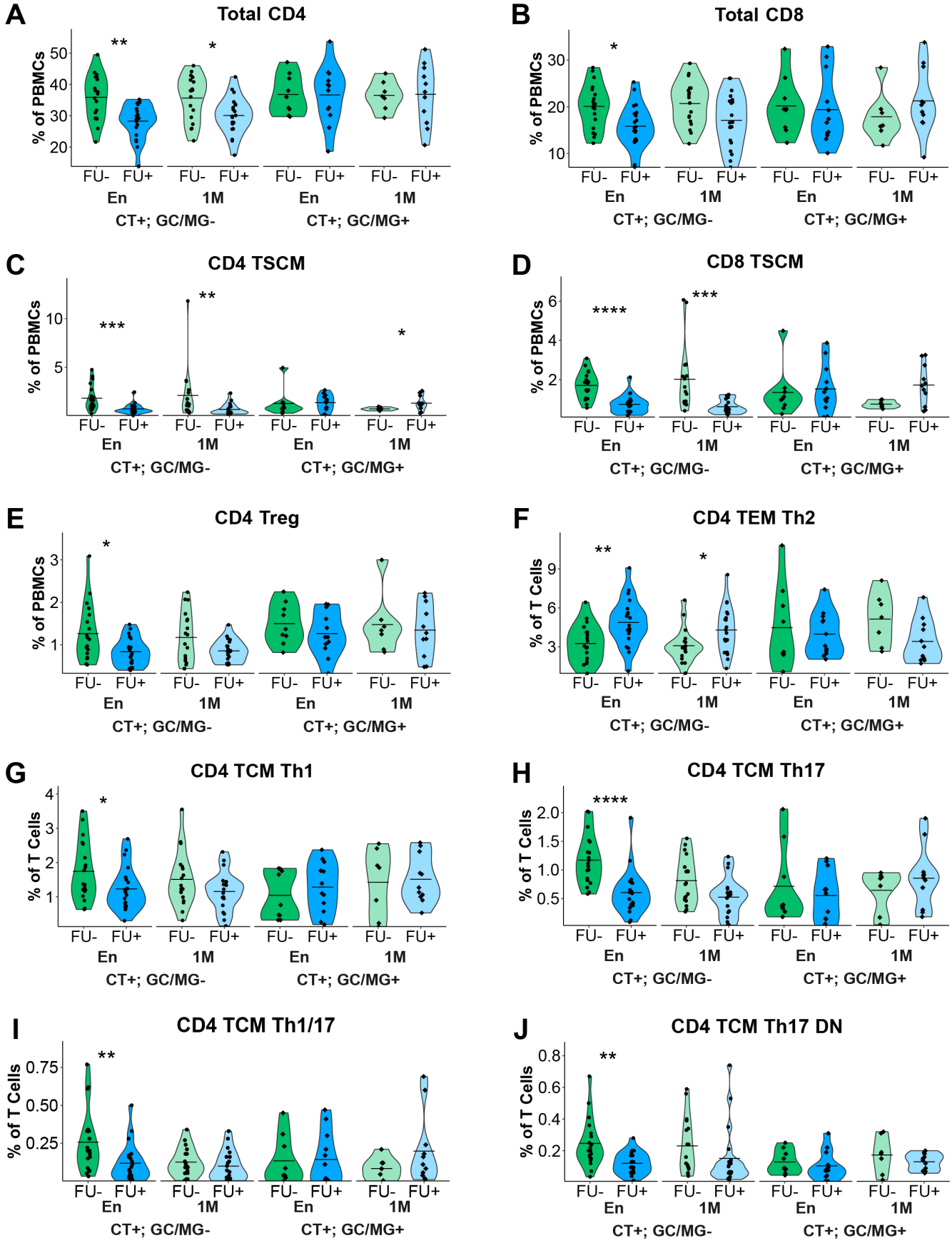

Figure 4. CD4 TCM Th1 and Th17 family subsets are enriched in peripheral blood of women without documented CT reinfection.

Frequencies of manually gated T cell subpopulations were stratified by follow-up negative (FU-) and follow-up positive (FU+) status. (A-J) T cell subpopulation percentages. Horizontal bar represents mean. Significance was determined by Wilcoxon rank-sum test. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.