Double positive CD4+CD8+ T cells are part of the adaptive immune response against Candida albicans

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1 **Double positive CD4+CD8+ T cells are part of the adaptive immune response against**

2 ***Candida albicans***

3

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13

14 **Running title:** CD4+CD8+ T cells reacts against *C. albicans* challenge

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17 **Abstract**

18 Although multiple immune cells participate in the innate and adaptive immune response
19 against *Candida albicans*, the elucidation of cellular and inflammation kinetics may be a
20 promising strategy to decipher events propitious to infection eradication. We used an *in vitro*
21 *Candida*-human leucocyte coculture approach to study the dynamics of rare CD4+CD8+
22 double positive T lymphocytes (DP T) produced in response to this fungus. Our results
23 highlight the presence of two phenotypically distinct subsets of DP T cells: CD4hiCD8lo and
24 CD4loCD8hi, and that the different ratio of these cells correlates with infection outcome. The
25 ratio of CD4hiCD8lo over CD4loCD8hi by day 6 was significantly higher in controlled
26 infections and decreased when infection persisted due to a significant increase in the
27 proportion of CD4loCD8hi. When infection was controlled, CD4hiCD8lo T cells secreted
28 IFNγ, TNFα, IL-4 and IL-10 cytokines two days after challenge. By day 2, under conditions
29 of persistent infection, CD4hiCD8lo and CD4loCD8hi T cells secreted significant levels of
30 IL-4 and IL-10, respectively, compared to uninfected cultures. Frequency kinetics and
31 original cytokine profiles detailed in this work indicate that DP T cells could participate in the
32 adaptive immune response to *C. albicans*.

33

34 Keywords: CD4+CD8+ double positive T cells, *C. albicans,* host-*Candida* interaction, tSNE

35 **1. Introduction**

36 *Candida albicans* is a lifelong colonizer of the human gastrointestinal tract that has evolved to
37 acquire a series of mechanisms to modulate human immune responses. These changes can
38 lead to large genetic, morphological and phenotypic variation [1–3]. Intensive research efforts
39 aimed at enhancing our understanding of anti-*Candida* immunity have identified multiple
40 immune cells involved in fungal sensing and defense [4–7]. Adaptive immunity against
41 *Candida* is complex and triggers a combination of T helper (Th1), Th2 and Th17 effector
42 cells engaged in the defense response against systemic or mucosal infections. IFNγ production
43 by Th1 cells increased the fungicidal activity of macrophages and neutrophils [8,9]. IL-17 and
44 IL-22 shaped Th17 responses and also induced the activation and recruitment of neutrophils
45 [10]. Th17 responses occur mainly throughout mucosal infections [11,12]. IL-4 and IL-10 are
46 associated with Th2 responses, and have different roles when responding to *Candida* [13,14].
47 Recent studies have shown that innate lymphoid cells (ILC) and innate-like lymphocyte
48 subpopulations (γδ T cells) may help to control fungal colonization and dissemination [15].
49 These results highlight some of the new challenges faced by researchers aiming to study of
50 the fine balance between T lymphocyte subsets for the development and maintenance of an
51 optimal anti-*Candida* response. Research efforts should be focused on the identification of T
52 cell effectors and the kinetics of cell activation and the cytokine production propitious to
53 infection resolution that does not cause deleterious effects within the host. Previous studies
54 have shown the existence of three subsets of stably double positive (DP) T cells in the blood
55 of individuals [16].

56 In the present study, we have determined the frequency phenotype and cytokine profile of
57 human DP T cells in response to *C. albicans* challenge and provide cytokine profiles, which
58 have the potential to contribute to the adaptive immune response against this fungal pathogen.

59 We captured these dynamic and complex interactions using a fungal persistence model
60 consisting of a human, delayed-type, multicellular reaction against *Candida* that had been
61 developed previously [17,18]. Our first observations suggested that an inadequate or
62 uncontrolled inflammatory response against *C. albicans* could fail to eradicate infection. T
63 lymphocyte subsets present at different timepoints post-challenge were identified. Based on
64 the exclusive expression of CD4 and CD8 co-receptors, we showed that the ratio of CD4+ to
65 CD8+ T lymphocytes was higher in *Candida*-leucocyte cocultures than in uninfected (UI)
66 controls. By analyzing this lymphoid compartment, we observed a significant increase in the
67 frequency of the expression of DP T cells six days after challenge.

68 While the majority of mature T cells leaving the thymus to secondary organs were exclusively
69 expressing only CD4+ or CD8+, low frequencies of CD4+CD8+ DP T cells (around 1% of
70 total T cells) could be detected in peripheral blood of healthy subjects [19]. The biological
71 significance of peripheral DP T cells is still not fully understood. Three subsets have been
72 described: CD4highCD8α low (CD4hiCD8lo), CD4lowCD8αβ high (CD4loCD8hi) and
73 CD4highCD8α low to intermediate (CD4hiCD8hi) [19–23]. Recent data has shown that a
74 fraction of CD4hiCD8lo identified by the co-expression of two chemokine receptors (CCR)
75 are regulatory T cells induced by gut microbiota [20]. In the context of the immune response
76 to a *Candida* infection, two studies have reported the presence of DP T cells. Ghaleb and
77 colleagues [24] detected DP T cells after vaginal inoculation of *C. albicans* into estrogen-
78 conditioned mice. Another study showed that the immunization of mice with mannan-HSA
79 conjugates from *C. dubliniensis* induced a significant rise in CD4+CD8+ T cells [25]. Based
80 on these observations, the present study examined the phenotype and the cytokine profile of
81 DP T cells in the context of an *in vitro* model of infection.

82

83

84 **2. Materials and Methods**

85 **2.1. *C. albicans* and human leucocytes co-cultures**

86 *C. albicans* clinical isolates (Caal93, Caal121 and Caal123) were provided by the
87 Parasitology and Medical Mycology Department, University of Nantes, France. One colony
88 from each clinical isolate was picked from YPD agar (1% yeast extract, 2% peptone, 2%
89 dextrose, 2% agar), transferred to YPD liquid medium and incubated overnight at 30°C in a
90 shaking incubator. After washing in PBS, 10^6 cells/ml blastoconidia were suspended in RPMI
91 1640 with 8% heat-inactivated pooled human serum (HS). Peripheral blood samples were
92 obtained from sixteen healthy volunteers by venipuncture. The gender of donors was 9
93 females and 7 males (aged between 22 and 50 years old). Research was carried out in
94 accordance with the Declaration of Helsinki. All subjects gave informed consent for research
95 testing. Three independent blood samples were obtained from each subject to evaluate the
96 intra and inter-individual variation of immune phenotypes. Peripheral blood mononuclear and
97 polynuclear cells were isolated by gradient density sedimentation, using LMS 1077
98 lymphocyte separation medium (PAA Laboratories, Austria). For coculture experiments,
99 human immune cells were adjusted to a final concentration of 10^6 cells/ml in RPMI medium,
100 supplemented with 8% HS. Yeast cells were added at a multiplicity of infection (MOI) ratio
101 of 2000:1 and incubated at 37°C, 5% CO_2 for the indicated time periods. *C. albicans*-
102 leucocyte cocultures were followed daily by light microscopy. Uninfected cells were used as
103 controls. Fungal growth into these cocultures was evaluated at different time points for each
104 clinical isolate. The candidacidal activity of human immune cells was measured by counting
105 the living yeasts by retrocultures on YPD agar plates through a colony-counting technique
106 (Colony-Forming Unit, CFU). The fungal burden was expressed by the mean of CFU/ml for
107 the three clinical isolates and the sixteen subjects.

108

109 **2.2. Flow cytometry analyses**

110 Cell culture supernatants were eliminated and leucocyte cocultures were washed twice in PBS
111 at 37°C. Cocultures were dispersed by pipetting and the total number of living cells at each
112 time point was assessed by cell counting in the presence of 0.5% eosin. The cells were
113 suspended in 200 μl of PBS 1% BSA and stained with a cocktail of fluorescent-conjugated
114 antibodies in PBS 0.1% BSA. The antibodies were specific to CD3-VioGreen (clone REA613,
115 dilution 1/11, MACS Miltenyi Biotec), CD4-VioBright-FITC (clone REA623, dilution 1/11,
116 MACS Miltenyi Biotec) and CD8-PE (clone REA734, dilution 1/50, MACS Miltenyi Biotec).
117 Stain specificity was verified with isotype-matched control antibodies VioBright-FITC-
118 conjugated IgG1, VioGreen-conjugated IgG1 and PE-conjugated IgG1. Cells were incubated
119 for 1h at 4°C in the dark, washed twice with PBS and analyzed by flow cytometry. After
120 single living cell gating, the mean percentages of viable cells vary between 75 to 95 % for all
121 samples. The kinetic of IFNγ, IL-17, IL-4, TNFα and IL-10 cytokine secretion by DP T cells
122 was also followed by flow cytometry (Miltenyi Biotec, Auburn, CA). After incubations with a
123 Catch Reagent for 5 min on ice and for 45 min at 37°C in warm (37°C) RPMI medium under
124 slow continuous rotation, cells were labeled with PE-conjugated IL-10 detection antibody (10
125 μl label/10^6 cells) and APC-conjugated IFNγ detection antibody (10 μl label/10^6 cells), PE-
126 conjugated IL-4 and APC-IL-17 detection antibody and PE-TNFα detection antibody.
127 Isolated cells were counterstained using VioBlue-labeled anti-CD8 antibody and PE-Vio770-
128 labeled anti-CD4 antibody. All data were acquired using a FACS LSRII instrument (BD
129 Biosciences) and analyzed with FlowJo software version 9.4.10 (Tree Star Inc.) and DIVA
130 software version 6.2 (BD Biosciences).

131

132 **2.3. T-distributed Stochastic Neighbor Embedding analyses**

133 Flow cytometer LSRII measured parameters were normalized and randomized. Data were
134 first analyzed using FlowJo software. After doublets, debris, and dead cells exclusions, living
135 cells were gated. Downsampling function of multiple intra- and interindividual measurements
136 was applied before data concatenation. Resulting FCS files were analyzed with the tSNE
137 module. Specific plots were generated to model global DP T cell immune responses to *C.*
138 *albicans* challenge. The following input settings were used before tSNE reduction: up to
139 300,000 concatenated cells per file, 1000 iterations, 20 perplexity, 0.5 Theta. Pairwise
140 comparations of all events were mapped in a low dimension space, arranging similar events
141 nearby and dissimilar cells farther away. Cells were clustered by CD4, CD8, CD4hiCD8lo,
142 CD4hiCD8hi and CD4loCD8hi. Specific tSNE analyses were realized comparing IFNγ, IL-17,
143 TNFα pro-inflammatory and IL-4 and IL-10 anti-inflammatory cytokines. Concatenated and
144 individual experimental data were identically gated in FlowJo and statistics were analyzed on
145 GraphPad Prism version 6.

146

147 **2.4. Statistics**

148 Statistical analyses were all carried out with Prism V6.0a software (GraphPad Software). The
149 fungal burdens after challenge with each *Candida* isolate were compared using a two-way
150 ANOVA test with Tukey's correction for multiple comparisons. For the quantification of
151 intra- and inter-subject variability at baseline, we pooled data from all individuals and we
152 used a data vector $X=\{x_{st}\}$ (individual s, time-point t) where each x_{st} was associated with a
153 subject and each subject had a total three measurements obtained from each of the time-
154 points. Then we used the ANOVA model to evaluate the total variance (total sum of squares),
155 subject-to-subject differences (between column variation) and the fraction of variance
156 explained by subject relative to the residual of the fit. The surface immune cell markers and
157 cytokine secreting cells were expressed in percentage. The inter-subject variability was

158 quantified within the columns as the sum of squares of differences between each subject and
159 the sixteen subjects mean. The post-challenge variability was quantified as the sum of squares
160 of the differences between the columns means and the grand mean. *P* values < 0.05 from
161 ANOVA model indicated significant differences in cytokine secretion frequencies compared
162 to the baseline (day 0). A F ratio was also calculated by computing respective *C. albicans*
163 strain to inter-subject mean square values in order to assess how these parameters influence
164 cytokine secretion. Large F ratio signified higher post-challenge variability than inter-subject
165 variability. The *P* value was determined from the F ratio and the two values of degrees of
166 freedom. The immune composition and cytokine kinetics of leucocyte cocultures was
167 analyzed by one-way ANOVA with Tukey's correction for multiple comparisons. *P* values of
168 ≤ 0.05 were considered significant.
169
170 **2.5. Ethics statement**
171 Healthy volunteers were recruited at the blood bank center (Etablissement Français du Sang
172 EFS, Pays de Loire, France). All subjects provided written informed consent for research
173 testing in accordance with the Declaration of Helsinki. A convention has been signed between
174 our laboratory (IICiMed, EA1155, Nantes Université) and the EFS (No. NTS-2013-02 and
175 CPDL-PLER-2018-015).
176
177 **3. Results and Discussion**
178 **3.1. Variation of DP T cell frequencies in healthy subjects**
179 Peripheral blood samples were obtained from sixteen healthy volunteers. Three blood samples
180 from each subject were obtained at three time points to evaluate intra- and inter-individual
181 variation of immune phenotypes. For the characterization of DP T cells, we first identified
182 lymphocyte cells among freshly isolated PBMC. T cells were identified after gating on CD3

183 positive cells, and then analyzed for the expression of CD4 and CD8. The frequencies of total
184 DP T cells and specific CD4loCD8hi, CD4hiCD8hi, and CD4hiCD8lo subsets were analyzed
185 in the CD3+ compartment (**Figure 1A**). Results revealed a wide range of DP T cell
186 frequencies on day 0 (**Figure 1B**). These populations showed the highest variability between
187 subjects (white boxes) and the lower variability within-subjects (gray boxes). The mean
188 frequencies of CD4loCD8hi, CD4hiCD8hi, and CD4hiCD8lo were 0.7 % ± 0.5, 0.15 % ±
189 0.10 and 0.9 % ± 0.6, respectively. No correlation was observed between DP T cell
190 frequencies and the donor gender. Interestingly, we observed a positive correlation between
191 the total DP T cell frequencies and the age of subjects (**Figure 1C**). A similar correlation was
192 previously determined using healthy individuals [23,26].
193

194 **3.2. Evolution of the fungal burden on leucocyte cocultures**

195 Caal93, Caal121 and Caal123 clinical isolates of *C. albicans* were cocultured with human
196 immune cells. *C. albicans* immune-infiltrate formation was observed daily using light
197 microscopy. Uninfected cells were used as controls. Between days four and six post challenge
198 with *C. albicans*, highly distinguishable, multicellular and multilayered immune-infiltrate
199 structures were formed in infected versus uninfected controls (**Figure 1D**). Proliferation of *C.*
200 *albicans* was assessed at specific time points post-challenge. According to previous
201 observations, phagocytes display high candidacidal activity throughout the first 3 days post-
202 infection in all types of *C. albicans* isolates. Afterward, surviving yeasts have been shown to
203 be responsible for a significant and rapid increase in the fungal burden occurring between 4
204 and 6 days post-challenge (**Figure 1E, G**). By day 6, fungal loads of Caal121 and Caal123
205 were significantly higher (760 ± 1357 and 1490 ± 1811 CFU/ml, respectively) than Caal93
206 (492 ± 1188 CFU/ml) ($p = 0.0011$).
207

208 **3.3. DP T cell kinetics vary post-challenge with *C. albicans***

209 Then, we tested whether the kinetics of DP T cells would vary after *C. albicans* challenge. To
210 address this question, analyses were performed by measuring fungal load six days post-
211 challenge. We applied a 100 CFU/ml cutoff on day 6 to assess the ability of each *C. albicans*
212 isolate to proliferate within stimulated leucocytes from each subject. This cutoff corresponded
213 to the mean CFU/ml used for leucocytes stimulation on day 0 (MOI 2000:1). Controlled
214 infection (CI, light grey) was defined by a fungal burden lower than this value, whereas a
215 persistent infection (PI, dark grey) was defined as a fungal burden that higher than this level.
216 Each subject-strain pair was classified as CI or PI based on this cutoff value. Overall, 89
217 subject-strain pairs were classified as PIs and 55 subject-strain pairs were classified as CIs.
218 Flow cytometry analyses of T lymphocyte subsets were then performed over time. As was
219 previously observed, the proportion of CD4+ T cells was stable over time. In contrast, the
220 proportion of CD8+ T cells was significantly reduced on day 6 after challenge compared to
221 day 0 (**Figure 1F**). DP T cells in the CD3+ compartment were analyzed in order to investigate
222 the origin of the relative reduction in CD8+ 6 days post-infection. Interestingly, we observed
223 that kinetics were different among clinical isolates of *C. albicans*. Hence, the mean
224 proportions of DP T cells were not significantly different than uninfected control between day
225 0 and 4, but the proportions were significantly increased when isolates Caal121 and Caal123,
226 under both CI and PI conditions, were compared to Caal93 and uninfected controls six d after
227 the initial challenge (**Figure 1H**, light and dark grey boxes). These findings are in accordance
228 with those reporting that a strong antigenic challenge, such as widespread viral or bacterial
229 infection, is associated with an increase in the proportion of DP T cells found within
230 peripheral blood samples [27]. As previously demonstrated in other work, our results also
231 suggest that phenotypic variability of clinical isolates of *C. albicans* may influence the fitness
232 of fungal populations [28].

233 **3.4. The ratio of CD4hiCD8lo over CD4loCD8hi cells is diminished in persistent C.**
234 **albicans infections**

235 We then analyzed the frequencies of DP T cells by day 6 by distinguishing between the three
236 subpopulations defined according to levels of CD4 and CD8 expression. We found no
237 significant difference over the time in the proportions of CD4hiCD8hi cells. The variations of
238 CD4hiCD8lo and CD4loCD8hi T cells frequencies were similar between UI, CI and PI until
239 the fourth day post *C. albicans* challenge, leading to similar CD4hiCD8lo and CD4loCD8hi
240 ratios (**Figure 2A**). Interestingly, this ratio increased by day 6 under CI and UI conditions due
241 to a significant reduction in the proportion of CD4loCD8hi cells, while it was reduced under
242 PI conditions due to a significant increase in the proportion of CD4loCD8hi cells (**Figure 2B**).
243 The frequencies of CD4loCD8hi cells were significantly higher than uninfected cells under
244 persistent conditions (**Figure 2C**).
245 We utilized multiple intra- and inter-subject measurements in order to generate a
246 concatenated spatial leucocyte differentiation map using non-linear dimensional reduction
247 tSNE analysis (tSNE X vs. tSNE Y). Multiple measurements from each healthy subject over
248 the time, and their single cell information from a 3-color flow cytometry panel and viability
249 dye, led to the clustering of major immune populations, CD4+CD8- (light blue), CD4-CD8+
250 (grey), and minor immune populations, CD4loCD8hi (red), CD4hiCD8lo (black) and
251 CD4hiCD8hi (green) cell subsets. We fitted a tSNE analysis according to UI, PI and CI
252 infection profiles. The plots produced by the tSNE analysis sorted the cells based on the
253 similarity of CD4+ and CD8+ expression markers, and thus their clustering under uninfected
254 and infected conditions. **Figure 2D** also highlights the high ratio of CD4hiCD8lo versus
255 CD4loCD8hi observed for CI compared to PI profiles. The tSNE analysis of DP T cells also
256 suggested substantial remodeling of cell subset phenotypes occurred throughout *Candida*
257 infection and showed that CD4loCD8hi cells were phenotypically similar to CD8+ cells and

258 CD4hiCD8lo cells were similar to CD4+ cells. These observations suggest that a diversity of
259 DP T cell phenotypes may occur in response to *C. albicans* challenge. Other experimental
260 studies have shown that peripheral DP T cells originate from CD4+ and CD8+ single-positive
261 cells re-expressing the other co-receptor in response to infectious and non-infectious stimuli
262 [12,29].

263 We then analyzed cytokine secretion profiles of DP T cells in order to elucidate the function
264 of these subpopulations post *C. albicans* challenge. The kinetics of IFNγ, IL-17, IL-4, TNFα
265 and IL-10 cytokine secretion were analyzed over time using flow cytometry. Cytokine
266 secretion by DP T cells did not significantly vary under uninfected conditions over the time as
267 reflected by lower F ratios (**Table 1**). Interestingly, CIs produced significantly higher F ratios
268 for IFNγ, TNFα, IL-4 and IL-10 compared to UI conditions, suggesting significant *C.*
269 *albicans* post-challenge variability over the inter-subject variability (**Table 1**). The global
270 variance of pro- and anti-inflammatory cytokines was due to a significant secretion by two
271 days compared to six days post-challenge (**Figure S1**). **Figure 2E-F** depicts frequencies of
272 cytokine secretion from CD4hiC8lo and CD4loC8hi T cells two days post-challenge, under
273 UI (white boxes), CI (light gray) and PI (dark gray) conditions. When *C. albicans* infection
274 was controlled (CI), CD4hiC8lo T cells secreted significantly higher levels of IFNγ and
275 TNFα pro-inflammatory cytokines two days post-challenge compared to uninfected cells (UI).
276 These cells also secreted significantly increased levels of IL-10 and IL-4 two days post-
277 challenge. CD4loCD8hi cells also secreted significant levels of IL-4 by day 2 and secretion of
278 TNFα was delayed, occurring by day 6 (**Figure S1**). During *C. albicans* persistence (PI), the
279 secretion of proinflammatory cytokines by both CD4hiCD8lo and CD4loCD8hi T cells was
280 lower and was not significantly different than levels measured under UI conditions (**Figure F,**
281 **Table 1**). Levels of IL-4 and IL-10 secreted by CD4hiCD8lo and CD4loCD8hi T cells,

282 respectively, were significantly increased when compared to levels secreted under UI
283 conditions.

284 Although, DP T cell subsets are specific for a diverse set of antigens from past, latent, and
285 persistent viral infections [19], our results suggest that they can also recognize fungal
286 pathogens. These observations suggest that human CD4loCD8hi and CD4hiCD8lo DP T cells
287 were responsive against *C. albicans* and could differentially secrete cytokines in response to
288 fungal pathogen challenge. Consistent with previous reports, our results suggest that
289 CD4hiCD8lo can drive secretion of IFNγ and TNFα cytokines [30,31]. The rapid secretion of
290 IL-10 may be necessary to dampen the pro-inflammatory effect of the IFNγ driven Th1
291 response [32]. Our observations also highlight that CD4hiCD8lo and CD4loCD8hi T cells
292 may display different cytokine profiles when the *C. albicans* infection persists. The biological
293 significance of tolerogenic CD4loCD8hi T cells during fungal persistence requires further
294 investigation. According to the kinetics and the original cytokine profiles of these cells, DP T
295 cells may participate in the adaptive immune response against *C. albicans*. By defining the
296 precise cellular orchestration of these cells throughout *Candida* infection, it may be possible
297 to manipulate immune responses and enhance effector function.

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302

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398

399 **Figure 1. DP T cells variability and *C. albicans*-leucocyte co-cultures.** (**A**) Gating strategy
400 for double-positive (DP) T cells. Peripheral blood samples were obtained from sixteen healthy
401 volunteers. Three independent blood samples from each subject were collected over three
402 months to analyze the intra- and inter-subject variabilities. PBMCs were isolated and stained
403 with specific antibodies. Human leucocytes were identified among freshly PBMC. Doublets
404 were excluded using forward scatter height (FSC-H) and forward scatter width (FSC-A). T
405 cells were identified within the CD3+ compartment (red gate) and analyzed for the expression
406 of CD4 and CD8 based on respective isotype controls. DP T cells were analyzed by gating
407 CD4loCD8hi, CD4hiCD8hi, and CD4hiCD8lo subsets in the same CD3+ compartment. (**B**)
408 Intra- and inter-subject variability of DP T cells. Multiple-measurement data from all subjects
409 we pooled with the data vector $X=\{x_{st}\}$ (individual s, time-point t), where each x_{st} was
410 associated with a subject and each subject had a total three measurements obtained from each
411 of the time-points. The ANOVA model was used to evaluate the total variance (total sum of
412 squares), subject-to-subject differences (between column variation) and the fraction of
413 variance explained by subject relative to the residual of the fit. (**C**) DP T cells frequency and
414 subject age correlation. (**D**) Light microscopy observation of representative immune-infiltrate
415 formation after six days after *C. albicans* challenge (Bar indicates 50 µm). (**E**) Fungal growth
416 into leucocyte cocultures. Human peripheral leucocytes were challenged with living yeasts of
417 Caal93, Caal121 and Caal123 *C. albicans* clinical isolates at a MOI phagocyte to yeast of
418 2000:1. The fungal burden evolution was followed at 0, 2, 4 and 6 days post-challenge days
419 by retro-culture and expressed as colony-forming units per ml (CFU/ml). The curve depicts
420 the mean + SD of the fungal burden of three separate experiments for each subject (16
421 subjects), each subject's cells with separate clinical isolate of *Candida* (each circle represents
422 144 data points). Uninfected cells were used as controls. Results were compared by a one-way
423 ANOVA test with Kruskal-Wallis correction for multiple comparisons. (**F**) Variations in

424 CD4+ and CD8+ T cells frequencies after *C. albicans* challenge. Box plots depict median,
425 minimum, and maximum percentages of immune cells according to UI (white boxes)
426 uninfected control, CI (light gray) and PI (dark gray) outcomes and on days 0, 2, 4 and 6 post-
427 challenge with *C. albicans*. (**G**) Fungal burden evolution among Caal93, Caal121 and
428 Caal123 clinical isolates of *C. albicans* (n = 16). (**H**) Variation in the percentages of total DP
429 T cells over the time and among clinical isolates of *C. albicans.* DP T cell frequencies were
430 analyzed in the CD3+ compartment. A cutoff of 100 CFU/ml by day 6 was applied to
431 determine the ability of each clinical isolate to proliferate into leucocyte cocultures. This
432 cutoff matches to the mean CFU/ml used for leucocytes stimulation on day 0. Controlled
433 infection (CI, light grey boxes) was referenced when the fungal burden was lower than this
434 cutoff and persistent infection (PI, dark grey boxes) when the fungal burden was higher.
435 Uninfected cells were used as controls (UI, white boxes). *, $p < 0.05$; **, $p < 0.001$; ***, $p <$
436 0.0001 (by one-way ANOVA with Tukey's multiple comparisons test).
437
438

439 **Figure 2. Variation of DP T proportions after *C. albicans* challenge.** (**A**) Total DP T cells
440 and specific CD4loCD8hi, CD4hiCD8hi, and CD4hiCD8lo subsets frequencies were analyzed
441 in the CD3+ compartment. CD4hiCD8lo over CD4loCD8hi ratio over the time was calculated
442 among persistent- (PI), controlled- (CI) infection profiles and UI controls. **(B)** Variation in the
443 percentages of CD4hiCD8lo and CD4loCD8hi T cells by days 0 and 6 after challenge among
444 persistent- (PI), controlled- (CI) infection profiles and UI controls. (**C**) Frequencies of
445 CD4loCD8hi T cells six days after challenge. **(D)** Representative flow-cytometry analysis
446 showing CD4hiCD8lo, CD4hiCD8hi and CD4loCD8hi T cells variation among PI, CI profiles
447 and UI controls. After normalization and randomization of flow cytometer measurements,
448 data were analyzed using FlowJo software by excluding doublets, debris, and dead cells and
449 gating on living cells. Downsampling function of multiple intra- and interindividual
450 measurements was applied before data concatenation. Resulting FCS files were analyzed with
451 the tSNE module. Specific plots were generated to model global DP T cell immune responses
452 to *C. albicans* challenge among PI, CI profiles and UI controls. Pairwise comparations of all
453 events were mapped in a low dimension space, sorting similar cells nearby and dissimilar
454 cells farther away. Cells were clustered by CD4 (light blue), CD8 (grey), CD4hiCD8lo
455 (black), CD4hiCD8hi (green) and CD4loCD8hi (red). **(E, F)** Similar flow cytometry staining
456 with a cocktail of fluorescent-conjugated antibodies specific to cytokines were performed to
457 analyze the cytokine secretion by DP T cells in response to *C. albicans* challenge. Specific
458 tSNE analyses were realized for IFNγ, IL-17, TNFα pro-inflammatory and IL-4 and IL-10
459 anti-inflammatory cytokines. Global cytokine secretion profiles were obtained for each
460 CD4hiCD8lo and CD4loCD8hi T cells among PI and CI profiles, and were compared to UI
461 controls. Box plots depict median, minimum, and maximum percentages of immune cells
462 according to UI (white boxes) uninfected control, CI (light gray) and PI (dark gray) outcomes

463 on day 2 after challenge with *C. albicans*. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; ****, p

464 < 0.00001 (by one-way ANOVA with Tukey's multiple comparisons test) ($n = 16$).

465

466 **Figure S1. Cytokine secretion by DP T after *C. albicans* challenge**. Cytokine secretion was
467 followed by flow cytometry. Specific tSNE analyzes were realized for IFNγ, IL-17, TNFα
468 pro-inflammatory and IL-4 and IL-10 anti-inflammatory cytokines. Global cytokine secretion
469 profiles were obtained for each CD4hiCD8lo and CD4loCD8hi T cells among PI, CI profiles
470 and were compared to UI controls. Box plots depict median, minimum, and maximum
471 percentages of immune cells according to UI (white boxes) uninfected control, CI (light gray)
472 and PI (dark gray) outcomes 6 days after challenge with *C. albicans*. *, $p < 0.05$; **, $p <$
473 0.001; ***, $p < 0.0001$; ****, $p < 0.00001$ (by one-way ANOVA with Tukey's multiple
474 comparisons test) (n = 16).
475
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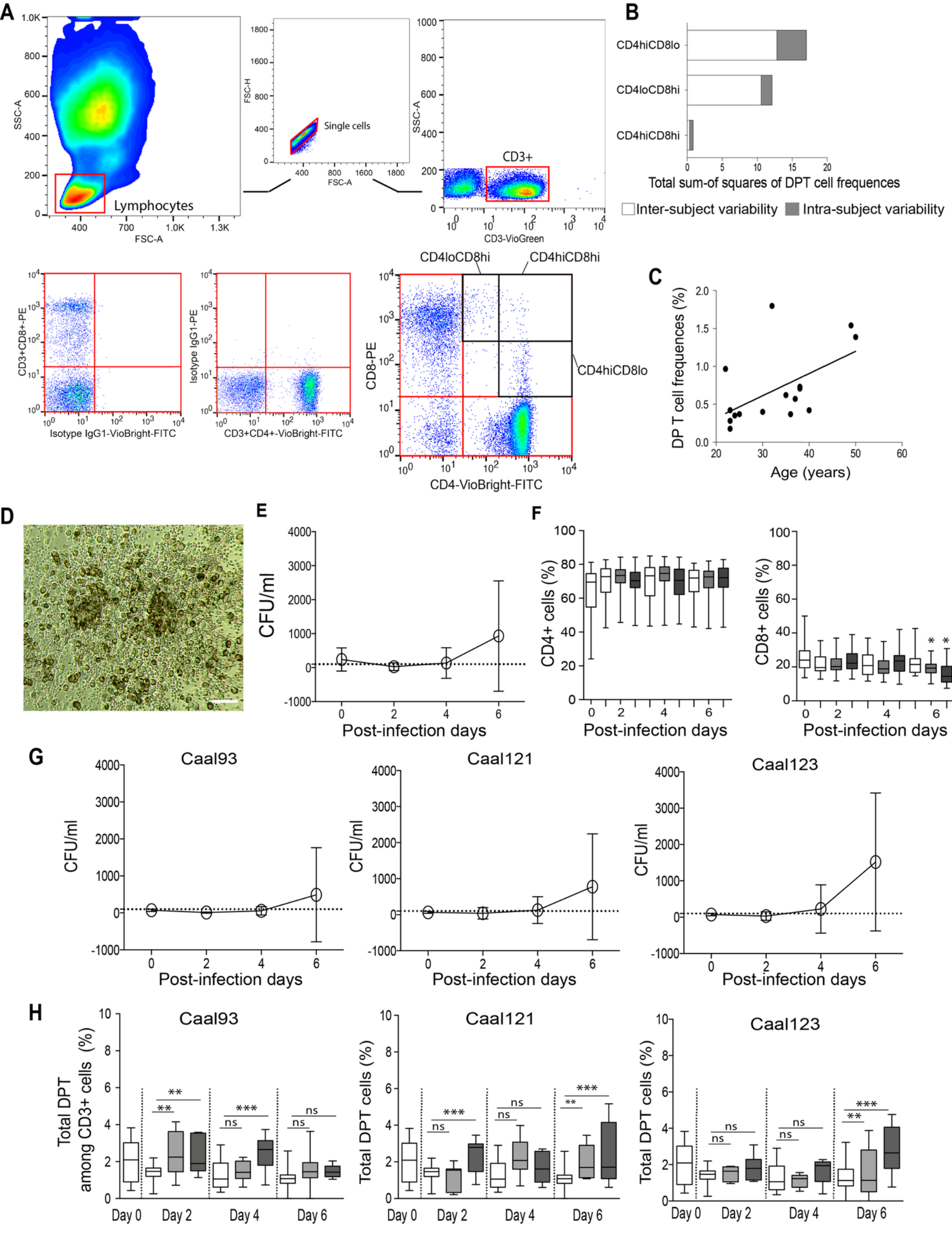
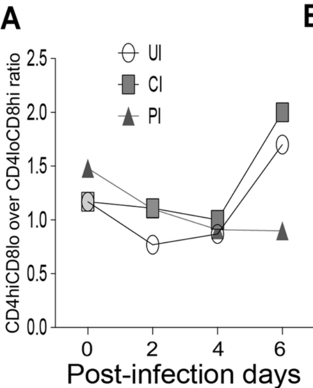
	DP T cells					
	UI		CI		PI	
	F ratio	*P*	F ratio	*P*	F ratio	*P*
IFNγ	1.32	0.3217	41.84	<0.0001	0.85	0.4400
TNFα	7.03	0.0031	12.82	0.0001	3.56	0.0440
IL-17	1.98	0.1625	1.95	0.1568	0.61	0.5463
IL-10	1.89	0.1937	28.61	<0.0001	3.34	0.0551
IL-4	2.39	0.1123	13.11	<0.0001	0.41	0.6665

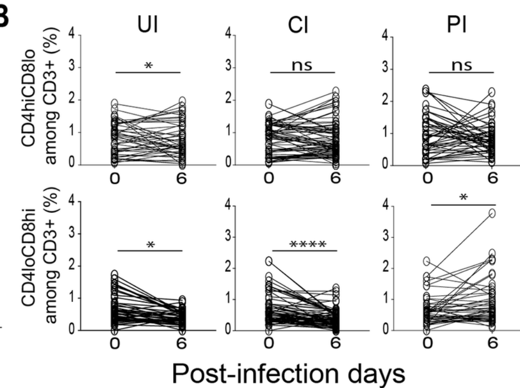
481 **Table 1:** One-way ANOVA analysis of the human cytokine secretion variability after
482 challenge by *C. albicans*. F: F ratio of post-challenge variability over inter-subject variability.
483 UI: Uninfected cells, CI: Controlled infection outcome, PI: Persistent-infection outcome. *P*
484 value: < 0.05 was significant.

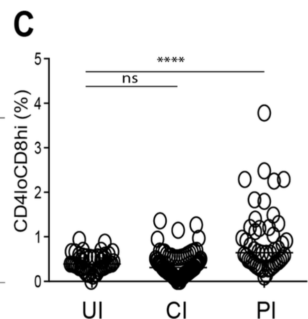
485



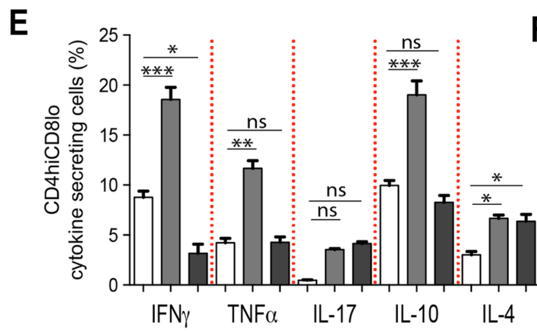
A
Lymphocytes
SSC-A
FSC-A
FSC-H
Single cells
CD3+
CD3-VioGreen
CD3+CD8+-PE
Isotype IgG1-VioBright-FITC
Isotype IgG1-PE
CD3+CD4+-VioBright-FITC
CD4loCD8hi
CD4hiCD8hi
CD4hiCD8lo
CD8-PE
CD4-VioBright-FITC
B
CD4hiCD8lo
CD4loCD8hi
CD4hiCD8hi
Total sum-of squares of DPT cell frequences
Inter-subject variability
Intra-subject variability
C
DP T cell frequences (%)
Age (years)
D
E
CFU/ml
Post-infection days
F
CD4+ cells (%)
CD8+ cells (%)
Post-infection days
G
Caal93
Caal121
Caal123
CFU/ml
Post-infection days
H
Caal93
Caal121
Caal123
Total DPT among CD3+ cells (%)
Total DPT cells (%)
Day 0
Day 2
Day 4
Day 6

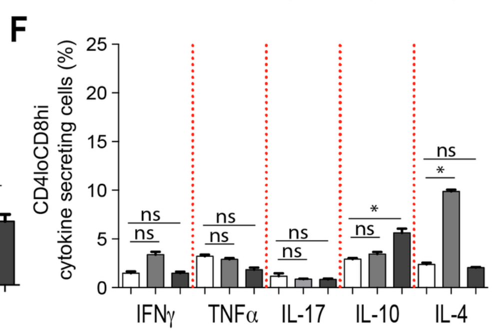

A
CD4hiCD8lo over CD4loCD8hi ratio
UI
CI
PI
Post-infection days


B
UI
CI
PI
CD4hiCD8lo among CD3+ (%)
CD4loCD8hi among CD3+ (%)
*
ns
ns
*
****
*
Post-infection days


C
CD4loCD8hi (%)
ns
****
UI
CI
PI

D
UI
CI
PI
CD8+
CD4+


E
CD4hiCD8lo cytokine secreting cells (%)
IFNγ
TNFα
IL-17
IL-10
IL-4


F
CD4loCD8hi cytokine secreting cells (%)
IFNγ
TNFα
IL-17
IL-10
IL-4