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

Barbara Misme-Aucouturier, Adel Touahri, Marjorie Albassier, Francine Jotereau, Patrice Le Pape, Nidia Alvarez-Rueda

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

Barbara Misme-Aucouturier[1], Adel Touahri[1], Marjorie Albassier[1], Francine Jotereau[2], Patrice Le Pape[1*] Nidia Alvarez-Rueda[1*].

[1] Nantes Université, CHU de Nantes, Cibles et médicaments des infections et du cancer, IICiMed, EA 1155, F-44000 Nantes, France.

[2] CRCINA, INSERM, Nantes Université, Nantes, France.

[*]Corresponding author:

E-mail: nidia.alvarez-rueda@univ-nantes.fr (NAR), patrice.le-pape@univ-nantes.fr (PLP).

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

Abstract

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

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

1. Introduction

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

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

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

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

2. Materials and Methods

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

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

2.2. Flow cytometry analyses

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

2.3. T-distributed Stochastic Neighbor Embedding analyses

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

2.4. Statistics

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

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

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

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

We then analyzed the frequencies of DP T cells by day 6 by distinguishing between the three subpopulations defined according to levels of CD4 and CD8 expression. We found no significant difference over the time in the proportions of CD4hiCD8hi cells. The variations of CD4hiCD8lo and CD4loCD8hi T cells frequencies were similar between UI, CI and PI until the fourth day post *C. albicans* challenge, leading to similar CD4hiCD8lo and CD4loCD8hi ratios (**Figure 2A**). Interestingly, this ratio increased by day 6 under CI and UI conditions due to a significant reduction in the proportion of CD4loCD8hi cells, while it was reduced under PI conditions due to a significant increase in the proportion of CD4loCD8hi cells (**Figure 2B**). The frequencies of CD4loCD8hi cells were significantly higher than uninfected cells under persistent conditions (**Figure 2C**).

We utilized multiple intra- and inter-subject measurements in order to generate a concatenated spatial leucocyte differentiation map using non-linear dimensional reduction tSNE analysis (tSNE X vs. tSNE Y). Multiple measurements from each healthy subject over the time, and their single cell information from a 3-color flow cytometry panel and viability dye, led to the clustering of major immune populations, CD4+CD8- (light blue), CD4-CD8+ (grey), and minor immune populations, CD4loCD8hi (red), CD4hiCD8lo (black) and CD4hiCD8hi (green) cell subsets. We fitted a tSNE analysis according to UI, PI and CI infection profiles. The plots produced by the tSNE analysis sorted the cells based on the similarity of CD4+ and CD8+ expression markers, and thus their clustering under uninfected and infected conditions. **Figure 2D** also highlights the high ratio of CD4hiCD8lo versus CD4loCD8hi observed for CI compared to PI profiles. The tSNE analysis of DP T cells also suggested substantial remodeling of cell subset phenotypes occurred throughout *Candida* infection and showed that CD4loCD8hi cells were phenotypically similar to CD8+ cells and

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

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

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

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

5. Funding

This work was supported by the Alliance de Recherche sur les Maladies Infectieuses (ARMINA), Région Pays de Loire 2012-2016, Grant number : 2012 09680 and the Ministère de l'Enseignement Supérieur et de la Recherche (France).

6. References

[1] Barnett JA. A history of research on yeasts 12: medical yeasts part 1, Candida albicans. Yeast 2008;25:385–417.

[2] Bougnoux ME, Diogo D, François N, Sendid B, Veirmeire S, Colombel JF, et al. Multilocus sequence typing reveals intrafamilial transmission and microevolutions of Candida albicans isolates from the human digestive tract. J Clin Microbiol 2006;44:1810–20.

[3] Fujimura KE, Sitarik AR, Havstad S, Lin DL, Levan S, Fadrosh D, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med 2016;22:1187–91.

[4] Wang X, van de Veerdonk FL, Netea MG. Basic Genetics and Immunology of Candida Infections. Infect Dis Clin North Am 2016;30:85–102.

[5] Jabra-Rizk MA, Kong EF, Tsui C, Nguyen MH, Clancy CJ, Fidel PL, et al. Candida albicans Pathogenesis: Fitting within the Host-Microbe Damage Response Framework. Infect Immun 2016;84:2724–39.

[6] Netea MG, Joosten LAB, van der Meer JWM, Kullberg B-J, van de Veerdonk FL. Immune defence against Candida fungal infections. Nat Rev Immunol 2015;15:630–42.

[7] Martinez-Alvarez JA, Perez-Garcia LA, Flores-Carreon A, Mora-Montes HM. The immune response against Candida spp. and Sporothrix schenckii. Rev Iberoam Micol 2014;31:62–6.

[8] Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, et al. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature 2012;484:514–8.

[9] Delsing CE, Gresnigt MS, Leentjens J, Preijers F, Frager FA, Kox M, et al. Interferon-gamma as adjunctive immunotherapy for invasive fungal infections: a case series. BMC Infect Dis 2014;14:166.

[10] Liang SC, Tan XY, Luxenberg DP, Karim R, Dunussi-Joannopoulos K, Collins M, et al. Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. J Exp Med 2006;203:2271–9.

[11] van de Veerdonk FL, Joosten LA, Shaw PJ, Smeekens SP, Malireddi RK, van der Meer JW, et al. The inflammasome drives protective Th1 and Th17 cellular responses in disseminated candidiasis. Eur J Immunol 2011;41:2260–8.

[12] Puel A, Cypowyj S, Bustamante J, Wright JF, Liu L, Lim HK, et al. Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. Science 2011;332:65–8.

[13] Mencacci A, Cenci E, Del Sero G, Fe d'Ostiani C, Mosci P, Trinchieri G, et al. IL-10 is required for development of protective Th1 responses in IL-12-deficient mice upon Candida albicans infection. J Immunol 1998;161:6228–37.

[14] Tavares D, Ferreira P, Arala-Chaves M. Increased resistance to systemic candidiasis in athymic or interleukin-10-depleted mice. J Infect Dis 2000;182:266–73.

[15] Spits H, Artis D, Colonna M, Diefenbach A, Di Santo JP, Eberl G, et al. Innate lymphoid cells--a proposal for uniform nomenclature. Nat Rev Immunol 2013;13:145–9.

[16] Parel Y, Chizzolini C. CD4+ CD8+ double positive (DP) T cells in health and disease. Autoimmun Rev 2004;3:215–20.

[17] Alvarez-Rueda N, Albassier M, Allain S, Deknuydt F, Altare F, Le Pape P. First human model of in vitro Candida albicans persistence within granuloma for the reliable study of host-fungi interactions. PLoS ONE 2012;7:e40185.

[18] Misme-Aucouturier B, Albassier M, Alvarez-Rueda N, Le Pape P. Specific Human and Candida Cellular Interactions Lead to Controlled or Persistent Infection Outcomes during Granuloma-Like Formation. Infect Immun 2017;85:e00807–16.

[19] Nascimbeni M, Shin E-C, Chiriboga L, Kleiner DE, Rehermann B. Peripheral CD4(+)CD8(+) T cells are differentiated effector memory cells with antiviral functions. Blood 2004;104:478–86.

[20] Godefroy E, Alameddine J, Montassier E, Mathé J, Desfrançois-Noël J, Marec N, et al. Expression of CCR6 and CXCR6 by Gut-Derived CD4+/CD8α+ T-Regulatory Cells, Which Are Decreased in Blood Samples From Patients With Inflammatory Bowel Diseases. Gastroenterology 2018;155:1205–17.

[21] Kerstein A, Müller A, Pitann S, Riemekasten G, Lamprecht P. Circulating CD4+CD8+ double-positive T-cells display features of innate and adaptive immune function in granulomatosis with polyangiitis. Clin Exp Rheumatol 2018;36 Suppl 111:93–8.

[22] Overgaard NH, Cruz JL, Bridge JA, Nel HJ, Frazer IH, La Gruta NL, et al. CD4+CD8β+ double-positive T cells in skin-draining lymph nodes respond to inflammatory signals from the skin. J Leukoc Biol 2017;102:837–44.

[23] Quandt D, Rothe K, Scholz R, Baerwald CW, Wagner U. Peripheral CD4CD8 double positive T cells with a distinct helper cytokine profile are increased in rheumatoid arthritis. PLoS ONE 2014;9:e93293.

[24] Ghaleb M, Hamad M, Abu-Elteen KH. Vaginal T lymphocyte population kinetics during experimental vaginal candidosis: evidence for a possible role of CD8+ T cells in protection against vaginal candidosis. Clin Exp Immunol 2003;131:26–33.

[25] Paulovičová E, Machová E, Tulinská J, Bystrický S. Cell and antibody mediated immunity induced by vaccination with novel Candida dubliniensis mannan immunogenic conjugate. Int Immunopharmacol 2007;7:1325–33.

[26] Ghia P, Prato G, Stella S, Scielzo C, Geuna M, Caligaris-Cappio F. Age-dependent accumulation of monoclonal CD4+CD8+ double positive T lymphocytes in the peripheral blood of the elderly. Br J Haematol 2007;139:780–90.

[27] Zuckermann FA. Extrathymic CD4/CD8 double positive T cells. Vet Immunol Immunopathol 1999;72:55–66.

[28] Schönherr FA, Sparber F, Kirchner FR, Guiducci E, Trautwein-Weidner K, Gladiator A, et al. The intraspecies diversity of C. albicans triggers qualitatively and temporally distinct host responses that determine the balance between commensalism and pathogenicity. Mucosal Immunol 2017;10:1335–50.

[29] Kitchen SG, Whitmire JK, Jones NR, Galic Z, Kitchen CM, Ahmed R, et al. The CD4 molecule on CD8+ T lymphocytes directly enhances the immune response to viral and cellular antigens. Proc Natl Acad Sci U S A 2005;102:3794–9.

[30] Desfrançois J, Moreau-Aubry A, Vignard V, Godet Y, Khammari A, Dréno B, et al. Double positive CD4CD8 alphabeta T cells: a new tumor-reactive population in human melanomas. PLoS ONE 2010;5:e8437.

[31] Parel Y, Aurrand-Lions M, Scheja A, Dayer JM, Roosnek E, Chizzolini C. Presence of CD4+CD8+ double-positive T cells with very high interleukin-4 production potential in lesional skin of patients with systemic sclerosis. Arthritis Rheum 2007;56:3459–67.

[32] Romani L. Immunity to fungal infections. Nat Rev Immunol 2011;11:275–88.

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

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

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

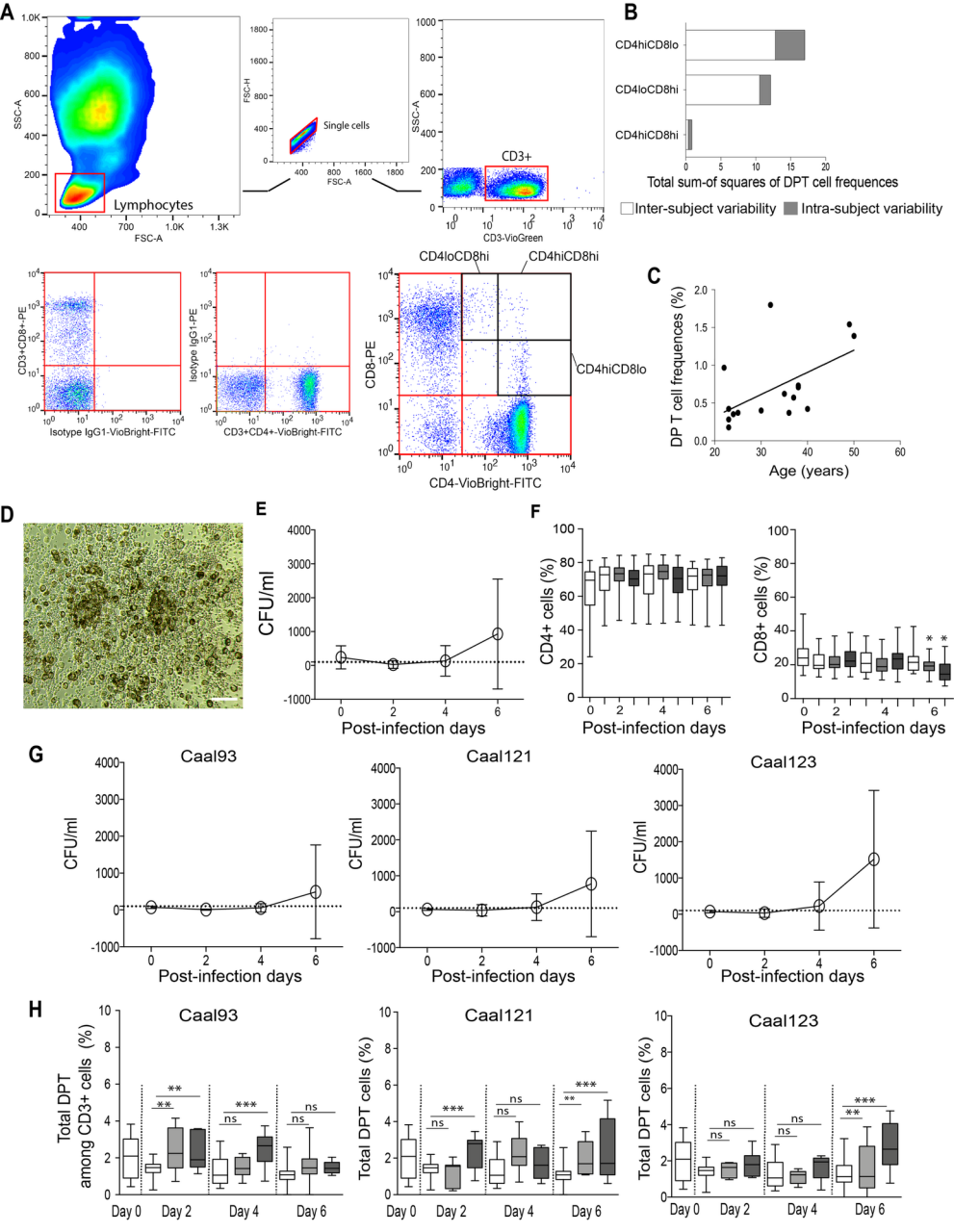
464 < 0.00001 (by one-way ANOVA with Tukey's multiple comparisons test) (n = 16).

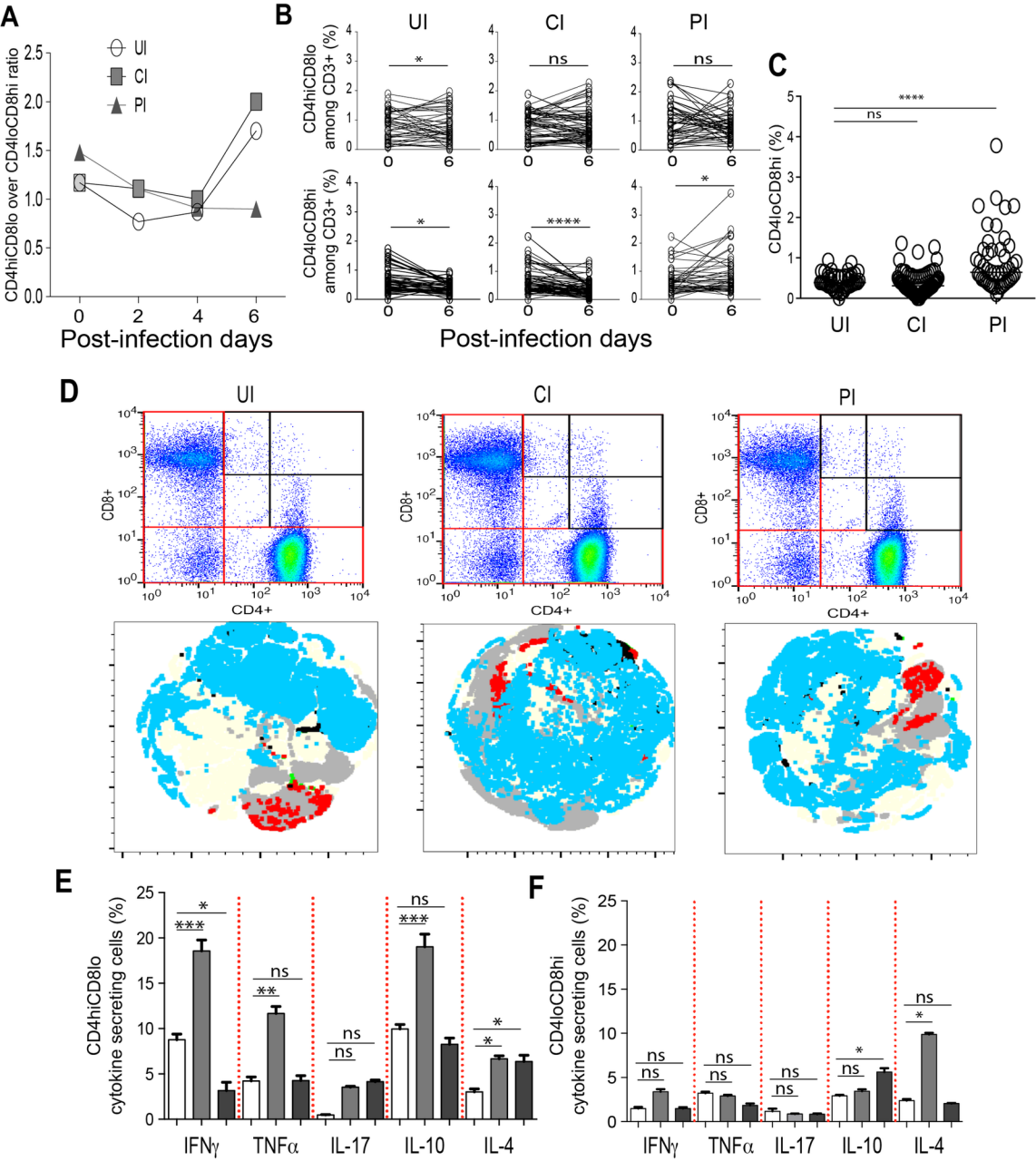
465

Figure S1. Cytokine secretion by DP T after *C. albicans* challenge. Cytokine secretion was followed by flow cytometry. Specific tSNE analyzes were realized for IFNγ, IL-17, TNFα pro-inflammatory and IL-4 and IL-10 anti-inflammatory cytokines. Global cytokine secretion profiles were obtained for each CD4hiCD8lo and CD4loCD8hi T cells among PI, CI profiles and were compared to UI controls. Box plots depict median, minimum, and maximum percentages of immune cells according to UI (white boxes) uninfected control, CI (light gray) and PI (dark gray) outcomes 6 days after challenge with *C. albicans*. *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$; ****, $p < 0.00001$ (by one-way ANOVA with Tukey's multiple comparisons test) (n = 16).

	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

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


A
SSC-A
FSC-A
Lymphocytes
FSC-H
Single cells
CD3+
CD3-VioGreen
B
CD4hiCD8lo
CD4loCD8hi
CD4hiCD8hi
Total sum-of squares of DPT cell frequences
Inter-subject variability
Intra-subject variability
CD3+CD8+-PE
Isotype IgG1-VioBright-FITC
Isotype IgG1-PE
CD3+CD4+-VioBright-FITC
CD4loCD8hi
CD4hiCD8hi
CD8-PE
CD4hiCD8lo
CD4-VioBright-FITC
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
Total DPT among CD3+ cells (%)
Total DPT cells (%)
Day 0
Day 2
Day 4
Day 6
ns


A
UI
CI
PI
CD4hiCD8lo over CD4loCD8hi ratio
Post-infection days
B
UI
CI
PI
CD4hiCD8lo among CD3+ (%)
CD4loCD8hi among CD3+ (%)
Post-infection days
C
CD4loCD8hi (%)
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