

Research Article

Clonal composition and persistence of antigen-specific circulating T follicular helper cells

Mengyun Hu^{1,4} , Samuele Notarbartolo^{1,5} , Mathilde Foglierini^{1,6} ,
Sandra Jovic¹, Federico Mele¹, David Jarrossay¹, Antonio Lanzavecchia²,
Antonino Cassotta^{#1}  and Federica Sallusto^{#1,3} 

¹ Institute for Research in Biomedicine, Università della Svizzera italiana, Bellinzona, Switzerland

² National Institute of Molecular Genetics, Milano, Italy

³ Institute of Microbiology, ETH Zurich, Zurich, Switzerland

⁴ Present address: Institute for Immunity, Transplantation and Infection, School of Medicine, Stanford University, Stanford, CA, USA

⁵ Present address: National Institute of Molecular Genetics, Milano, Italy

⁶ Present address: Service d'immunologie et d'allergie, CHUV, Lausanne, Switzerland

T follicular helper (T_{FH}) cells play an essential role in promoting B cell responses and antibody affinity maturation in germinal centers (GC). A subset of memory CD4⁺ T cells expressing the chemokine receptor CXCR5 has been described in human blood as phenotypically and clonally related to GC T_{FH} cells. However, the antigen specificity and relationship of these circulating T_{FH} (cT_{FH}) cells with other memory CD4⁺ T cells remain poorly defined. Combining antigenic stimulation and T cell receptor (TCR) V β sequencing, we found T cells specific to tetanus toxoid (TT), influenza vaccine (Flu), or *Candida albicans* (*C.alb*) in both cT_{FH} and non-cT_{FH} subsets, although with different frequencies and effector functions. Interestingly, cT_{FH} and non-cT_{FH} cells specific for *C.alb* or TT had a largely overlapping TCR V β repertoire while the repertoire of Flu-specific cT_{FH} and non-cT_{FH} cells was distinct. Furthermore, Flu-specific but not *C.alb*-specific PD-1⁺ cT_{FH} cells had a “GC T_{FH}-like” phenotype, with overexpression of *IL21*, *CXCL13*, and *BCL6*. Longitudinal analysis of serial blood donations showed that Flu-specific cT_{FH} and non-cT_{FH} cells persisted as stable repertoires for years. Collectively, our study provides insights on the relationship of cT_{FH} with non-cT_{FH} cells and on the heterogeneity and persistence of antigen-specific human cT_{FH} cells.

Keywords: *Candida albicans* · influenza vaccine · T follicular helper cells · TCR V β sequencing · tetanus toxoid



Additional supporting information may be found online in the Supporting Information section at the end of the article.

Correspondence: Federica Sallusto
e-mail: sallustf@ethz.ch

[#]These authors contributed equally to the work.

© 2022 The Authors. *European Journal of Immunology* published by Wiley-VCH GmbH

www.eji-journal.eu

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.

Introduction

Among memory T helper cells, T follicular helper (T_{FH}) cells have been considered as a distinct subset due to their unique ability to promote B cell responses and antibody affinity maturation in germinal centers (GCs) [1–4]. While T_{FH} cells by definition are fully differentiated effector cells, studies have shown that they are also able to generate long-lived memory cells residing in lymphoid tissues [5–8]. In humans, a subset of T helper cells with constitutive expression of the T_{FH} signature chemokine receptor CXCR5 was found in peripheral blood and termed as circulating T_{FH} (cT_{FH}) cells [1, 2, 9]. cT_{FH} cells represent a heterogeneous population, with some cells being phenotypically closer to GC T_{FH} than others. In the context of infections or vaccinations, frequency of $CCR7^{lo}PD-1^{+}$ cT_{FH} cells able to promote antibody production was shown to correlate with active T_{FH} cell differentiation in secondary lymphoid organs [10]. Furthermore, frequency of $PD-1^{+}CXCR3^{-}$ cT_{FH} cells correlates positively with the humoral immune response against HIV-1 [11] and HIV-1-specific $ICOS^{+}PD1^{+}$ cT_{FH} cells expressing several GC T_{FH} -related genes are induced upon HIV-1 vaccination [12]. $ICOS^{+}PD1^{+}$ cT_{FH} cells are also found upon influenza vaccination and their frequency correlates with the antibody responses [13–15].

The origin of blood cT_{FH} cells and their relationship with T_{FH} cells found in lymphoid tissues or other body fluids remains still poorly defined and is currently subject to intense investigation. Repertoire analysis by TCR V β sequencing has revealed clonal overlap between bulk populations of blood cT_{FH} and tonsillar T_{FH} cells, thus providing first evidence of a direct link between the two anatomically distinct subsets [12, 16]. A recent study analyzing the composition of T cells in human efferent lymphatic vessels identified a $CXCR5^{+}PD-1^{+}$ T_{FH} population with an intermediate phenotype between GC T_{FH} cells and $ICOS^{+}PD-1^{+}CD38^{+}$ cT_{FH} cells [17]. Lymph T_{FH} cells might represent a circulatory intermediate between GC T_{FH} and blood cT_{FH} , in line with a “ T_{FH} on the move” model of cell trafficking across different lymph nodes through the lymphatic and blood systems [18]. The fact that only a proportion of blood cT_{FH} cells shares functional properties with *bona fide* GC T_{FH} raises the question whether all cT_{FH} cells are directly related to T_{FH} cells that participated in the GC reactions in lymphoid organs.

In this study, we focused our analysis on antigen-specific memory cT_{FH} cells induced in vivo by pathogens or vaccines. We found that cT_{FH} and non- cT_{FH} cells specific for *Candida albicans* (*C.alb*) or tetanus toxoid (TT) were functionally diverse but had a largely overlapping TCR V β repertoire. In contrast, cT_{FH} specific for influenza antigens (Flu) had a distinct TCR V β repertoire compared to non- cT_{FH} cells and, when expressing PD-1, phenotypically resembled GC T_{FH} . In-depth longitudinal TCR V β sequencing analysis of serial blood samples unraveled the persistence and stability of Flu-specific cells, indicating long-lived cT_{FH} and non- cT_{FH} cell responses. Collectively, our findings demonstrate that human cT_{FH} cells are a heterogeneous population and their clonal relatedness with other T helper cell subsets and persistence depend upon their antigen specificity.

Results

The clonal relationship between cT_{FH} and non- cT_{FH} cells depends on the nature of the antigen

To define the antigen-specificities of human cT_{FH} cells and non- cT_{FH} cells, we sorted $CD4^{+}$ memory T cells from PBMCs of healthy donors and divided them into four subsets based on the expression of CXCR5 and CCR6 (Fig. 1A and Fig. S1). T cells were labeled with CFSE and stimulated with autologous monocytes pulsed with Flu, TT or *C.alb* antigens; frequency of antigen-reactive CFSE^{low} proliferating cells was analyzed after 5–6 days, as previously described [19]. While TT-specific T cells were present in all subsets, Flu-specific and *C.alb*-specific T cells were present mainly in the CCR6[−] or CCR6⁺ subsets, respectively (Fig. 1B), consistent with previous data [20, 21]. Within the CCR6[−] and CCR6⁺ subsets, *C.alb*-specific T cells were significantly more abundant in the non- cT_{FH} (CCR6⁺CXCR5[−]) subset while Flu-specific T cells were significantly more abundant in the cT_{FH} (CCR6[−]CXCR5⁺) subset. In all cases, cT_{FH} cells produced lower amounts of effector cytokines (i.e. IFN- γ , IL-4, IL-17, and IL-22) than non- cT_{FH} cells in response to antigenic stimulation (Fig. S2). Collectively, these data indicate that both cT_{FH} and non- cT_{FH} subsets contain memory T cells specific for common pathogens or vaccines and that, among the antigens tested, influenza virus antigens are able to induce a high frequency of cT_{FH} cells.

The finding that cT_{FH} and non- cT_{FH} cells responded to different extents to antigenic stimulations prompted us to further investigate their lineage relationship. Using a previously established workflow [19, 22], we isolated CFSE^{low} cells from the antigen-stimulated cultures and performed next-generation sequencing to quantify and compare TCR V β clonotypes of different antigen-specific T cell populations. As shown in Fig. 1C, the number of clonotypes was high in the T cell subsets best responding to antigenic stimulation, reflecting a diverse and polyclonal repertoire. Clonotype sharing between cT_{FH} and non- cT_{FH} cells was however diverse, with large overlaps in the case of TT and *C.alb* but limited overlap in the case of Flu, in particular between CCR6[−] cT_{FH} and CCR6[−] non- cT_{FH} cells that were the main responding subsets to Flu (Fig. 1C, D). Dendrogram plots based on the Morisita-Horn similarity index between pairs of TCR V β repertoires showed a close clonal relationship between *C.alb*-specific cT_{FH} and non- cT_{FH} cells and between TT-specific cT_{FH} and non- cT_{FH} cells. In contrast, in the case of Flu a closer clonal relationship was detected between CCR6⁺ and CCR6[−] cT_{FH} subsets and between CCR6⁺ and CCR6[−] non- cT_{FH} subsets (Fig. 1E). Pair-wise comparisons of the TCR V β clonotype frequency distributions further demonstrated that a large proportion of the expanded clonotypes were shared between cT_{FH} and non- cT_{FH} cells responding to *C.alb* or TT, while highly expanded Flu-specific clonotypes were not shared and unique to either cT_{FH} or non- cT_{FH} cell subset (Fig. 1F, G).

To further corroborate the above findings, we introduced internal controls by performing TCR V β sequencing of technical replicates. Extensive clonotype sharing was measured among replicates of non- cT_{FH} cell cultures, which was also evident, although

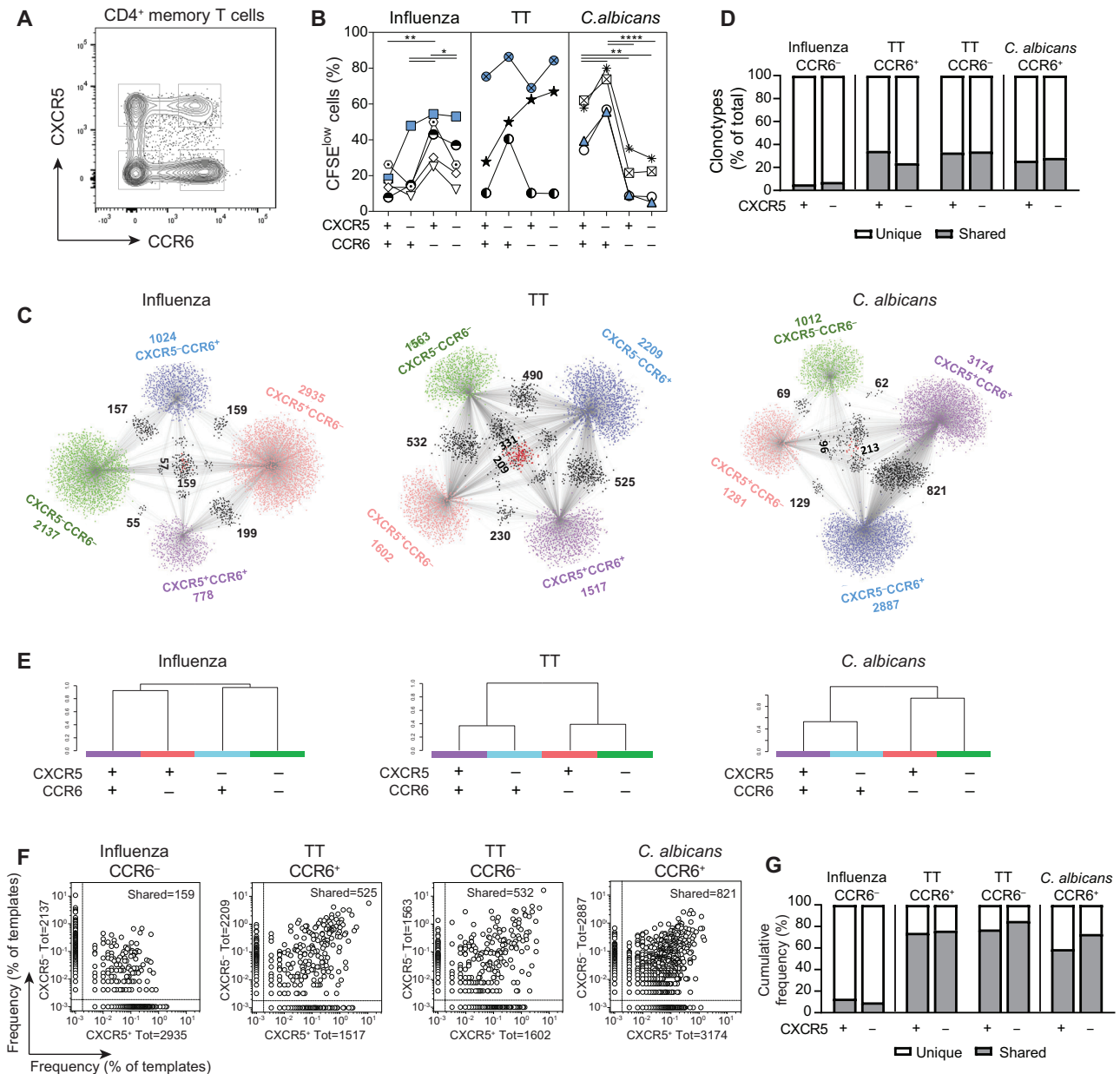


Figure 1. Variable TCR V β repertoire overlap between cTFH and non-cTFH cells specific for different microbial antigens. (A) Flow cytometry analysis on a representative donor showing the expression of CXCR5 and CCR6 on CD4⁺ memory T cells. (B) Four memory T cell subsets were FACS-sorted according to CXCR5 and CCR6 differential expression, labeled with CFSE, and stimulated with influenza vaccine (Flu), tetanus toxoid (TT) or *C. albicans* (*C. alb*) in the presence of autologous monocytes. Shown are the percentages of proliferating CFSE^{low} cells at 6–7 days post stimulation. In all conditions, 85%–98% of CFSE^{low} cells expressed the activation markers CD25 and/or ICOS. Each symbol indicates a different healthy donor (n = 5 donors for Flu, n = 3 for TT, and n = 4 for *C. alb*). * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.001, **** p-value < 0.0001, as determined by two-tailed paired t test. (C) Flu-, TT- and *C. alb*-specific CFSE^{low} T cells from each subset were FACS-sorted and their repertoire was analyzed by next generation TCR V β sequencing. Shown are the cytoscape clonal networks of TCR V β repertoire of antigen-specific T cells isolated from CXCR5⁺CCR6⁺ (purple), CXCR5⁺CCR6⁻ (blue), CXCR5⁻CCR6⁻ (pink), and CXCR5⁻CCR6⁺ (green) T cell subsets. Numbers of total (colored) and shared (black) TCR V β clonotypes are indicated. Clonotypes shared among two or three subsets are shown as black dots and clonotypes shared among all four subsets are shown as red dots. Data are from three representative donors (one for each antigen condition, indicated in panel B with blue symbols) out of six donors analyzed. (D) Shown is the fraction of unique and shared TCR V β clonotypes in each sample reported in panel C. Only the major responder T cell subsets for each type of antigen are reported. (E) Dendrogram plots of repertoire similarity among the four subsets based on the Morisita-Horn similarity index between pairs of TCR V β repertoires. (F) Pairwise comparisons of TCR V β clonotype frequency distribution in antigen-specific CFSE^{low} T cell lines isolated from cTFH and non-cTFH cells reported in panel C. Frequencies are reported as a percentage of productive templates. The total number of clonotypes is indicated in the x- and y-axes. The number of clonotypes shared between two samples are reported in the upper right corner. (G) Shown is the cumulative frequency of unique and shared TCR V β clonotypes in each sample reported in panel C. Only the major responder T cell subsets for each type of antigen are reported.

to a lower extent, among replicates of cT_{FH} cell cultures (Fig. S3A–D). Data normalization based on the repertoire overlap between CXCR5⁺ technical replicates confirmed the lower overlap in Flu-specific cT_{FH} and non-cT_{FH} repertoires compared to *C.alb*-specific cT_{FH} and non-cT_{FH} cells (Fig. S3E). Collectively, these data indicate that the overlap of TCR V β repertoires of antigen-specific cT_{FH} and non-cT_{FH} cells is variable and depending on the nature of the antigen.

Influenza-specific cells are enriched in PD-1⁺ cT_{FH} and have a distinct repertoire

Previous studies have shown that PD-1 is expressed on nearly 30% of cT_{FH} cells and that PD-1⁺ cT_{FH} cells, which are also Ki67, CD38, and HLA-DR negative [10, 11], are close to GC T_{FH} cells in terms of phenotype and function [10–12, 14, 23–25]. Based on these earlier findings, we set out to perform antigenic stimulation of cT_{FH} cells sorted according to PD-1 expression (Fig. S1B). Flu-specific cells were present at higher frequencies in the PD-1⁺ cT_{FH} subset compared to the PD-1[−] cT_{FH} and non-cT_{FH} subsets, whereas the opposite was observed for *C.alb*-specific cells (Fig. 2A). For both specificities, the PD-1⁺ cT_{FH} subset produced very little of any of the cytokines tested (Fig. S4). TCR V β repertoire analysis showed that PD-1⁺ cT_{FH} cell clonotypes were poorly shared with PD-1[−] cT_{FH} and non-cT_{FH} cell clonotypes in Flu- but not *C.alb*-specific response (Fig. 2B–D). Accordingly, Flu-specific PD-1⁺ cT_{FH} and non-cT_{FH} cells had the lowest normalized TCR overlap score while *C.alb*-specific PD-1⁺ cT_{FH} and PD-1[−] cT_{FH} cells had the highest (Fig. 2E).

To further corroborate the above findings, we selected the top 5% expanded clonotypes (i.e. 433 Flu-specific and 201 *C.alb*-specific clonotypes) and calculated a dendrogram of repertoire relatedness based on the Morisita-Horn similarity analysis (Fig. 2F). As expected, technical replicates of each subset clustered together with the exception of *C.alb*-specific PD-1⁺ cT_{FH}, whose repertoire largely overlapped with PD-1[−] cT_{FH} cells. Conversely, Flu-specific PD-1⁺ cT_{FH} displayed a distinct TCR V β repertoire characterized by low overlap with Flu-specific PD-1[−] cT_{FH} and non-cT_{FH} populations.

Collectively, these data demonstrate a differential distribution of antigen-specific cells in the PD-1⁺ and PD-1[−] cT_{FH} cell subsets. Flu-specific T cells were preferentially found in the PD-1⁺ cT_{FH} subset, which is considered to be closely related to *bona fide* GC T_{FH} cells.

Influenza-specific PD-1⁺ cT_{FH} cells phenotypically resemble GC T_{FH} cells

To further investigate the phenotype of antigen-specific PD-1⁺ cT_{FH}, PD-1[−] cT_{FH}, and non-cT_{FH} cells avoiding unintended changes that may be caused by prolonged in vitro culture, we adopted a protocol [26] to isolate antigen-specific cells based on surface overexpression of CD40L shortly after antigenic stimula-

tion (Fig. S5A). Briefly, PD-1⁺ cT_{FH}, PD-1[−] cT_{FH}, and non-cT_{FH} cells were stimulated with antigen-pulsed autologous monocytes in the presence of CD40-blocking antibodies. After 24 hours, activated T cells that upregulated CD40L were sorted and subject to Nanostring nCounter analysis to quantify the transcripts of 579 immune-relevant genes. By analyzing the genes that were differentially expressed between PD-1⁺ cT_{FH} and PD-1[−] cT_{FH}, and further comparing those transcripts between Flu- and *C.alb*-stimulated cells, we identified 40 genes that were up-regulated in PD-1⁺ cT_{FH} cells in both antigen-stimulated cultures, including *HLA-DR*, *CXCL13*, *IL17F*, *LAG3*, *ENTPD1* (*CD39*), *CD80*, *IFNG* (Fig. S5B, C). Interestingly, transcripts of 49 genes were uniquely upregulated in Flu-specific PD-1⁺ cT_{FH}, including *BCL6* and *IL21*. Furthermore, nine genes that were upregulated in Flu-specific PD-1⁺ cT_{FH} were downregulated in *C.alb*-specific PD-1⁺ cT_{FH} (fig. S5B–D).

By focusing on selected transcripts of interest, we found that Flu-specific PD-1⁺ cT_{FH} were enriched for a GC T_{FH} gene signature, including overexpression of *IL21*, *CXCL13*, *BCL6*, *SLAMF*, *BTLA*, *TIGIT*, *SH2D1A*, while Th1-related genes, such as *CXCR3*, *TBX21*, *PRDM1* (*BLIMP-1*), were expressed at low levels (Fig. 3A). Notwithstanding the expression of *CXCL13*, *SLAMF6* and a few other GC T_{FH}-related genes, the enrichment of GC T_{FH} signature was less evident in *C.alb*-specific PD-1⁺ cT_{FH} compared to Flu-specific PD-1⁺ cT_{FH} cells. As expected, Th17-related genes were preferentially expressed by *C.alb*-specific non-cT_{FH} cells and were mostly negatively enriched in PD-1⁺ and PD-1[−] cT_{FH} (Fig. 3A). Ranking GC T_{FH}-related genes based on the ratio of their expression in PD-1⁺ cT_{FH} to PD-1[−] cT_{FH}, (Fig. 3B), we found that *CXCL13* was the most preferentially overexpressed transcript in both Flu- and *C.alb*-specific PD-1⁺ cT_{FH} cells, consistent with the notion that secretion of high amounts of CXCL13 is an attribute of GC T_{FH} cells [27–29]. PD-1⁺ cT_{FH} cells were also able to secrete high amounts of CXCL13 in the culture supernatant upon antigenic stimulation (Fig. 3C). Interestingly, transcripts for IL-21, a key signature cytokine secreted by T_{FH} cells and promoting B cell proliferation, class switch recombination and plasma cell differentiation [30–32], were markedly overexpressed only in Flu-specific PD-1⁺ cT_{FH} (4.3 fold) but not in *C.alb*-specific PD-1⁺ cT_{FH} cells (1.34 fold). The differential expression of *IL21* mRNA was further confirmed at the protein level by measuring IL-21 concentration in the day 7-stimulated culture supernatants (Fig. 3D).

Collectively, these data demonstrate that Flu-specific PD-1⁺ cT_{FH} cells overexpress several GC T_{FH}-related genes compared to PD-1[−] cT_{FH} cells. The enrichment for a GC T_{FH} gene signature is less evident in *C.alb*-specific cT_{FH} cells, thus revealing that the level of cT_{FH} polarization towards *bona fide* GC T_{FH} phenotype is antigen-dependent.

Influenza-specific cT_{FH} and T_{EM} cells are persistent and phenotypically stable

To comprehensively measure the relationships and evolution of antigen-specific cT_{FH} and non-cT_{FH} cell repertoires, we performed

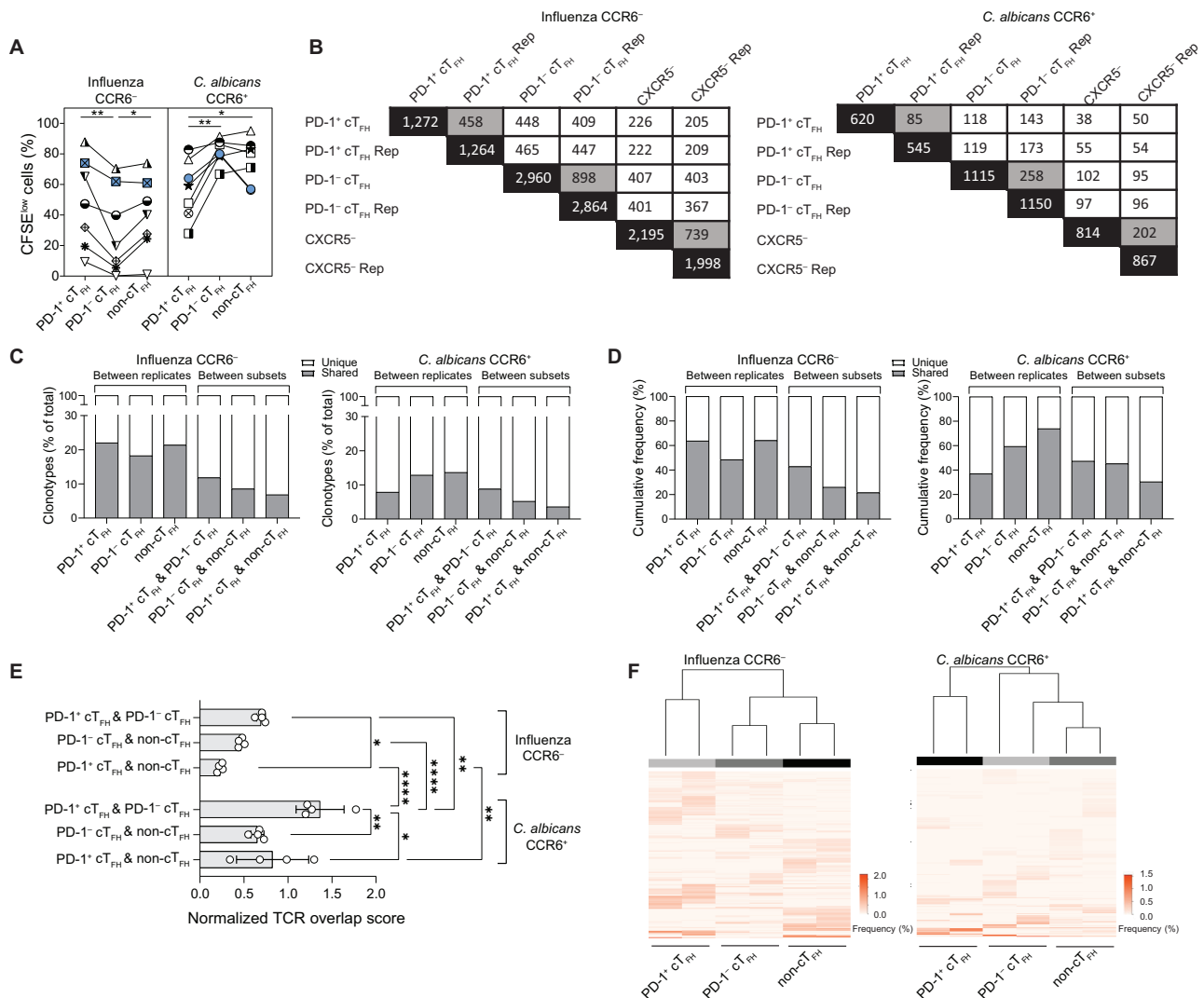


Figure 2. Flu-specific cells are enriched in PD-1⁺ cT_{FH} and have a distinct repertoire. (A) Percentages of proliferating CFSE^{low} cells measured at day 6–7 post stimulation of T cell subsets with Flu or *C. alb.* in the presence of autologous monocytes. Each symbol indicates a different healthy donor ($n = 7$ for Flu, and $n = 7$ for *C. alb.*). * p -value < 0.05 , ** p -value < 0.01 , as determined by two-tailed paired t test. (B) The TCR V β repertoire of Flu- and *C. alb.*-specific CFSE^{low} T cells from each subset was determined by next generation sequencing. The numbers of TCR V β clonotypes detected from each antigen-specific PD-1⁺ cT_{FH}, PD-1⁻ cT_{FH} and CXCR5⁻ subsets and technical replicates are reported in the black cells of the table. The numbers of clonotypes shared between technical replicates are shown in the grey cells. The numbers of clonotypes shared between any pair of two different subsets are reported in the white cells. Data are from two representative donors (one for each antigen condition, indicated in panel A with blue symbols) out of three donors analyzed. (C) Percentages of unique and shared clonotypes between technical replicates and between different subsets, determined by the Jaccard index calculated on the samples reported in panel B. (D) Cumulative frequencies of unique and shared clonotypes between technical replicates and between different subsets, determined as the average of the cumulative frequencies of the shared clonotypes in each of the two subsets calculated on the samples reported in panel B. (E) Normalized TCR overlap score between pairs of TCR V β repertoires of antigen-specific PD-1⁺ cT_{FH}, PD-1⁻ cT_{FH} and non-cT_{FH} cells, calculated by the Chao-Jaccard similarity index normalized on technical replicates. Data are multiple pairwise comparisons of the samples reported in panel B. * p -value < 0.05 , ** p -value < 0.01 , *** p -value < 0.001 , **** p -value < 0.0001 , as determined by one-way ANOVA with Tukey multiple comparisons test. (F) Heat map reporting the frequencies of the top 5% most expanded TCR V β clonotypes in the samples reported in panel B (433 Flu-specific and 201 *C. alb.*-specific clonotypes, respectively) ranked by the sum frequency of each clone. Dendrograms are based on the Morisita-Horn similarity index between pairs of TCR V β repertoires.

a longitudinal TCR V β repertoire analysis by collecting at multiple time points large blood donations from a healthy donor. At each time-point, we FACS-sorted from PBMCs cT_{FH} and non-cT_{FH} memory cells, which for these experiments were further divided into central memory (T_{CM}) and effector memory (T_{EM}) subsets based on CCR7 expression (Fig. S1C). The experiment was designed

taking into account our previous work showing that Influenza-specific T cells are present in all three memory subsets [33] and that CCR6 is expressed on both T_{CM} and T_{EM} subsets [34]. The CFSE-labelled T cell subsets were then stimulated with Influenza vaccine and the TCR V β repertoire of proliferating CFSE^{low} T cells was performed by next-generation sequencing, as described pre-

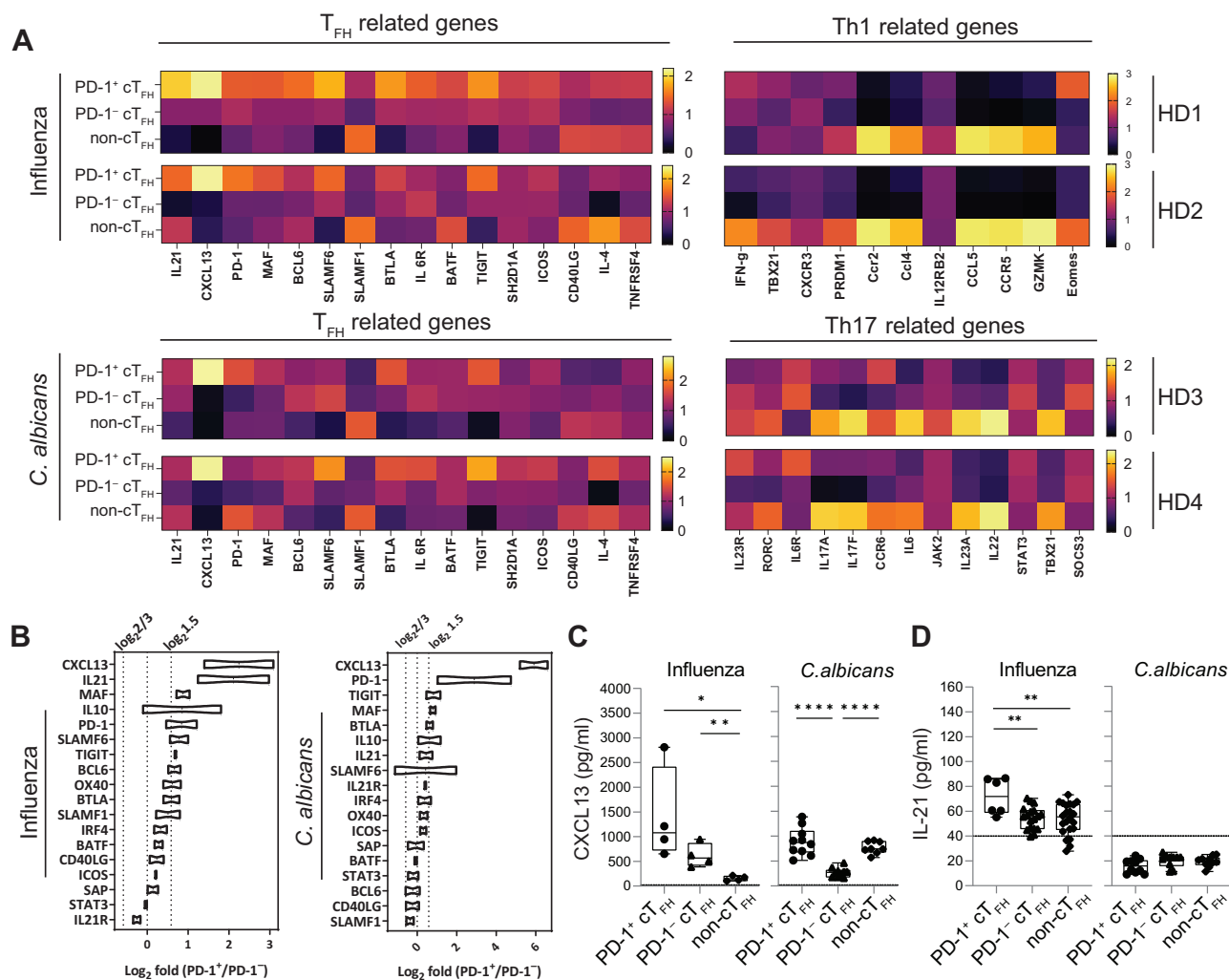


Figure 3. Flu-specific PD-1⁺ cTFH cells phenotypically resemble GC TH cells. (A) Heat map of the mRNA expression level of selected genes from antigen-specific PD-1⁺ cTFH, PD-1⁻ cTFH, and non-cTFH cells. TH-related and Th1-related genes are shown for Flu-specific cells (upper panels). TH and Th17-related genes are shown for *C. albicans*-specific cells (lower panels). Gene expression is normalized based on the average mRNA counts of each gene from the three T cell subsets. Data from n = 4 healthy donors (HD1-4) are shown. (B) Shortlist of GC TH-related genes ranked based on the gene expression ratio of PD-1⁺ cTFH to PD-1⁻ cTFH specific for influenza (left panel) or *C. albicans* (right panel). Ratios falling into the range of 0.67–1.5 were considered not significant. (C) Concentrations of CXCL13 in the day 6–7 culture supernatants of T cell subsets stimulated with Flu or *C. alb.* as measured by ELISA. For each antigen condition, shown are the experimental replicates (n = 4–10) from one representative donor out of n = 3 donors analyzed. Background cytokine production measured in T cells cultured with monocytes in the absence of the antigen is shown as dashed lines. * p-value < 0.05, ** p-value < 0.01, *** p-value < 0.0001, as determined by two-tailed unpaired t test. (D) Concentrations of IL-21 in the day 6–7 culture supernatants of T cell subsets stimulated with Flu or *C. alb.* as measured by ELISA. For each antigen condition, shown are the experimental replicates (n = 6–20) from one representative donor out of n = 3 donors analyzed. Background cytokine production measured in T cells cultured with monocytes in the absence of the antigen is shown as dashed lines. ** p-value < 0.01, as determined by two-tailed unpaired t test.

viously. Using this method, we identified at the initial time-point 3529, 2900, and 3090 TCR V β clonotypes in Flu-specific cTFH, T_{CM}, and T_{EM} cultures, respectively (Fig. 4A). Pairwise comparison of TCR V β clonotype frequency distributions in CFSE^{low} cultures from different subsets revealed extensive sharing of the most expanded clonotypes, particularly between Flu-specific T_{CM} and T_{EM} (Fig. 4A, left). As shown in the Venn diagram in Figure 4A, hundreds of TCR V β clonotypes were found in common between different T cell subsets, with 318 clonotypes found shared among all three subsets (Fig. 4A, right). TCR V β repertoire overlap was particularly high between Flu-specific T_{CM} and T_{EM} as compared

to cTFH at most time-points of analysis, as measured by the Chao-Jaccard index of similarity (Fig. 4B).

To get insights on the evolution of the clonal architecture of antigen-specific T cell repertoires, we followed Flu-specific TCR V β clonotypes in samples spanning a period of 42 months. The total number of Flu-specific TCR V β clonotypes detected from different T cell subsets was consistently high at each time point analyzed, spanning from 2196 to 7920 clonotypes (Fig. 4C) and demonstrating a polyclonal T cell response sustained by vaccination or infection. We then selected the whole TCR V β repertoires of CFSE^{low} Flu-specific cultures at the initial time-point (t1,

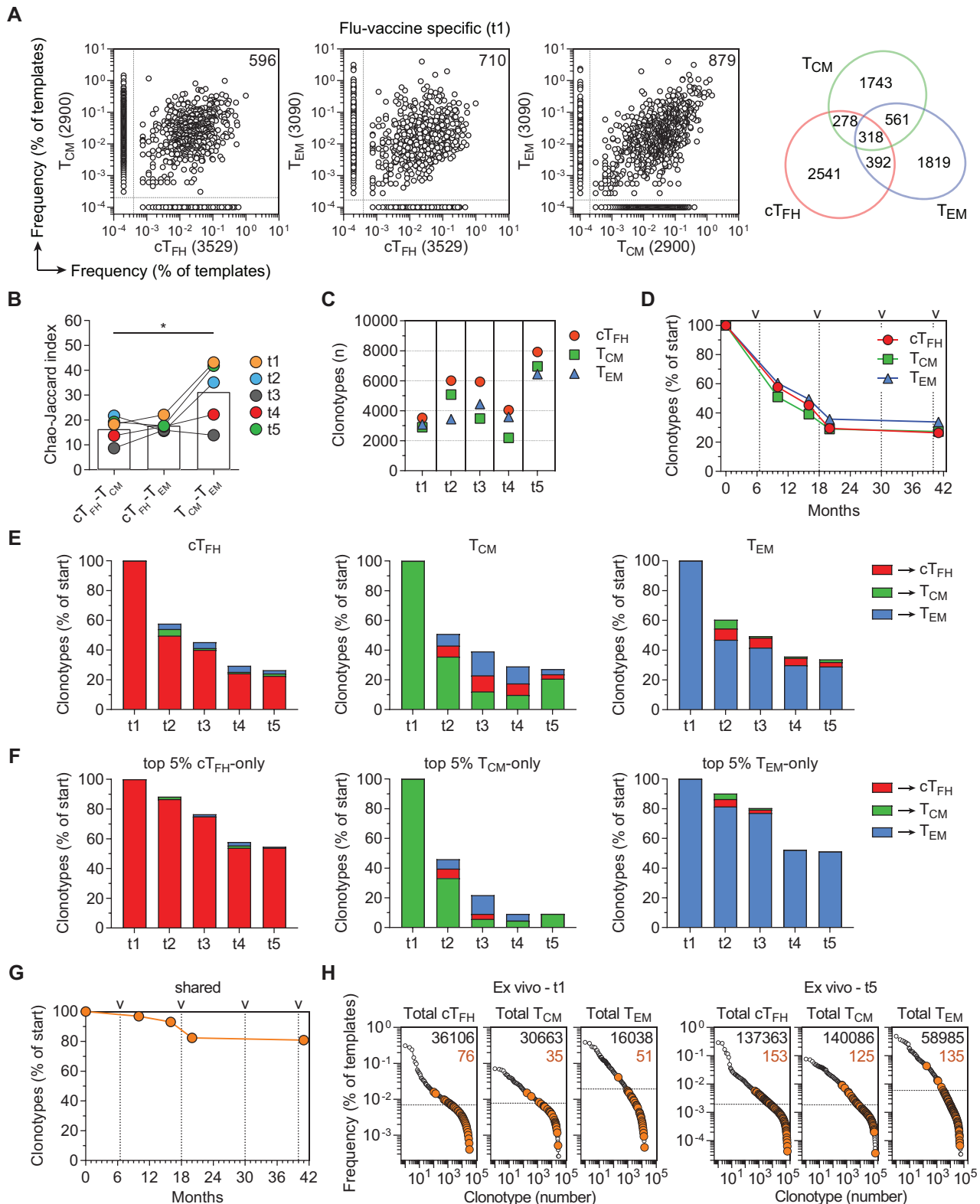


Figure 4. Flu-specific cT_{FH} and T_{EM} cells induced by infection or vaccination are persistent and phenotypically stable. (A) Serial blood donations from a Flu vaccinated individual were collected and next generation TCR V β sequencing was performed on CFSE^{low} proliferating T cells isolated from Flu-stimulated cT_{FH} , T_{CM} , and T_{EM} subsets (initial input 5×10^5 cells per subset) at each time-point. Shown is the pairwise comparison of TCR V β clonotype frequency distribution in Flu-specific cT_{FH} , T_{CM} , and T_{EM} subsets at the initial time-point of analysis (t1: May 2014). Frequencies are shown as percentage of productive templates. The total number of clonotypes is indicated in the x- and y-axes. Values in the upper right corner represent the number of clonotypes shared between two samples. The Venn diagrams show the number of unique and shared Flu-specific TCR

composed by 3529, 2900, and 3090 clonotypes in cT_{FH} , T_{CM} and T_{EM} , respectively), tracked their clonotypes in the CFSE^{low} fractions obtained at subsequent time-points (Fig. 4D) and quantified the proportion of the t1 TCR V β clonotypes transitioning towards other subsets at each time-point (Fig. 4E). Flu-specific clonotypes from cT_{FH} and T_{EM} were mostly detected in the original subset at all time points, whereas a high frequency of Flu-specific T_{CM} clonotypes were found in the cT_{FH} and T_{EM} subsets and became undetectable in the T_{CM} subset at later time points (Fig. 4E).

At the initial time-point (t1) the Flu-specific TCR V β repertoires comprised many low frequency-clonotypes found uniquely in one subset and not shared with others, and a few highly expanded clonotypes that were shared among cT_{FH} , T_{CM} and T_{EM} (Fig. 4A, right). To better decipher the role of each of those components to the overall stability and evolution of Flu-specific repertoires, we selected and followed longitudinally each of them. In particular at the t1, 2541 clonotypes were found uniquely in cT_{FH} (hereafter named “ cT_{FH} -only”), 1743 uniquely in T_{CM} (“ T_{CM} -only”), and 1819 uniquely in T_{EM} (“ T_{EM} -only”), that corresponded to 72%, 60% and 59% of the Flu-specific TCR V β clonotypes from cT_{FH} , T_{CM} and T_{EM} at t1, respectively. To exclude potential biases in detection due to low expansion, we selected the top 5% expanded t1 TCR V β clonotypes of each class (127 clonotypes for cT_{FH} -only cells, 87 for T_{CM} -only, and 91 for T_{EM} -only) and checked for their presence in the CFSE^{low} cultures from subsequent time points (Fig. 4F). A strong persistence was observed for Flu-specific t1 clonotypes from cT_{FH} -only and T_{EM} -only, with almost 50% of them being detected throughout 42 months. Most cT_{FH} -only and T_{EM} -only t1 clonotypes were preferentially found again at each subsequent time-point in the original subset (Fig. 4F), indicating rare transitions toward other subsets during the analysis timeframe. Conversely, Flu-specific t1 clonotypes from T_{CM} -only showed a

drop in detection in subsequent time-points, which is consistent with the contraction of certain clonotypes below the sampling and sequencing detection limits. In line with the longitudinal analysis of the whole subset, also T_{CM} -only clonotypes showed a higher proportion of transitions toward other subsets (Fig. 4F), thus indicating phenotypic plasticity of Flu-specific T_{CM} cells.

At the initial time-point (t1), 318 TCR V β clonotypes were found shared among cT_{FH} , T_{CM} , and T_{EM} . When followed longitudinally, the vast majority (>80%) of those heterogeneous clonotypes were detected again in the CFSE^{low} fractions from subsequent time points' samples (Fig. 4G, left). Comparison with the TCR V β repertoire of total cT_{FH} , T_{CM} , and T_{EM} sequenced directly ex-vivo revealed that those shared clonotypes were clonally expanded at both the initial (t1) and the final time-point (t5) of analysis (Fig. 4H, right), thus indicating the existence of a small pool of expanded and functionally heterogeneous Flu-specific T cells that persist in vivo.

Discussion

Several studies in humans have established the connections between T_{FH} cells in secondary lymphoid organs and cT_{FH} in blood, providing evidence that T_{FH} cells that have been engaged in GC reaction in B cell follicles can then enter the circulation and possibly persist as memory cT_{FH} cells [16–18]. Whether all cT_{FH} are derived from GC T_{FH} remains however to be defined. Our study suggests that not all blood cT_{FH} cells may be the descendent of GC T_{FH} cells and that some cells are closer to blood non- cT_{FH} cells. Several lines of evidence support this notion. Firstly, the TCR V β sequencing data indicate substantial repertoire overlap

V β clonotypes between cT_{FH} , T_{CM} and T_{EM} subsets. (B) Sample overlap between pairs of Flu-specific TCR V β repertoires was calculated using the Chao–Jaccard similarity index. The bar histograms show the Chao–Jaccard index between pairs of Flu-specific cT_{FH} , T_{CM} , and T_{EM} TCR V β repertoires obtained from serial blood donations of a healthy donor (t1: May 2014, t2: Feb 2015, t3: Sep 2015, t4: Jan 2016, t5: Nov 2017). * $p < 0.05$ as determined by two-tailed paired t test. (C) Number of productive TCR V β clonotypes detected by next generation sequencing of Flu-specific cT_{FH} (red circles), T_{CM} (green squares), and T_{EM} (blue triangles) cells at each time-point of the longitudinal analysis. (D and E) The Flu-specific TCR V β clonotypes found at the initial time-point (t1) in CFSE^{low} cultures from cT_{FH} (3529 clonotypes), T_{CM} (2900 clonotypes), and T_{EM} (3090 clonotypes) were followed in a longitudinal analysis of serial blood donations. The fraction of TCR V β clonotypes detected in CFSE^{low} cultures at each subsequent time-point is reported in (D) as percentage of start for cT_{FH} (red circles), T_{CM} (green squares), and T_{EM} (blue triangles). Dates of seasonal Flu vaccination are reported as dashed lines. The stacked barplots in (E) show a longitudinal analysis of the phenotype distribution of Influenza-specific clonotypes originally detected at the initial time-point (t1) in CFSE^{low} cultures from T cell subsets (cT_{FH} plotted on the left, T_{CM} in the center, T_{EM} on the right). Each sector reports the percentage of TCR V β clonotypes that are found within the original subset (cT_{FH} , T_{CM} and T_{EM} for the plots on the left, center and right, respectively) or transitioned to other subsets at each time-point. Data are expressed as percentage of start, and subsets are color-coded (cT_{FH} in red, T_{CM} in green, and T_{EM} in blue). (F) Within the Flu-specific TCR V β clonotypes found at the initial time-point (t1) only in CFSE^{low} cultures from cT_{FH} (cT_{FH} -only: 2541 clonotypes), T_{CM} (T_{CM} -only: 1743 clonotypes), or T_{EM} (T_{EM} -only: 1819 clonotypes), the top 5% clonotypes were shortlisted (top 5% cT_{FH} -only: 127 clonotypes, top 5% T_{CM} -only: 87 clonotypes, top 5% T_{EM} -only: 91 clonotypes) and followed in CFSE^{low} cultures from subsequent time-points. The stacked barplots report a longitudinal analysis of the phenotype distribution of the shortlisted TCR V β clonotypes detected at each subsequent time-point (top 5% cT_{FH} -only plotted on the left, top 5% T_{CM} -only in the center, top 5% T_{EM} -only on the right). Each sector reports the percentage of TCR V β clonotypes that are found within the original subset (cT_{FH} , T_{CM} and T_{EM} for the plots on the left, center and right, respectively) or transitioned to other subsets at each time-point. Data are expressed as percentage of start, and subsets are color-coded (cT_{FH} in red, T_{CM} in green, and T_{EM} in blue). (G and H). The Flu-specific TCR V β clonotypes found at the initial time-point (t1) shared among CFSE^{low} cultures from cT_{FH} , T_{CM} and T_{EM} were shortlisted (318 clonotypes) and followed in subsequent time-points. The fraction of TCR V β clonotypes detected at each subsequent time-point is reported in (G) as percentage of start. Dates of seasonal Flu vaccination are reported as dashed lines. Panel (H) shows the frequency distribution of TCR V β clonotypes from total cT_{FH} , T_{CM} , and T_{EM} subsets sequenced directly after ex vivo isolation from the same individual at t1 (May 2014, plots on the left) and t5 (Nov 2017, plots on the right). Frequencies are shown as percentage of productive templates. Colored circles mark the TCR V β clonotypes belonging to the shortlist of 318 shared clonotypes. The total number of TCR V β clonotypes and the number of retrieved clonotypes from the group of 318 shared clonotypes are reported on top of each graph in black and orange, respectively. Dotted lines in the graphs indicate the frequency threshold of the top 5% expanded clonotypes.

between *C.alb*- or TT-specific cT_{FH} and non-cT_{FH} cells. Secondly, longitudinal TCR V β sequencing showed that a fraction of cells from the CXCR5⁺ T_{CM} subset were able to convert into CXCR5⁺ cT_{FH} cells. Moreover, antigen-specific cT_{FH} cells can produce the same types of cytokines as the non-cT_{FH} counterparts, albeit to a different extent. On this regard, previous reports have highlighted the plasticity of the T_{FH} gene expression program by showing that *ex-vivo* sorted lymphoid PD-1⁺ T_{FH} cells could be polarized in vitro to produce IFN- γ , IL-4, and IL-17, and likewise Th1, Th2, and Th17 cells, could be reprogrammed to acquire T_{FH} features [35]. Collectively, previous studies and our new data suggest that cT_{FH} cells are a heterogeneous T cell population with different origins.

In the context of influenza vaccination, a population of activated cT_{FH} cells characterized by the expression of ICOS, PD-1 and CXCR3 has been shown to emerge in the blood at day 7 post-vaccination and to correlate with antibody response [10, 13–15]. Although in two of these studies and in our own investigation cT_{FH} cells were not sorted based on CXCR3 expression, it is likely that a large proportion of Flu-specific T cells overexpress CXCR3 [36], thus suggesting that Flu-specific CXCR3⁺ PD-1⁺ cT_{FH} cells are closely related to GC T_{FH} cells. However, previous reports monitoring the response to HIV-1 [11] or malaria infection [24] proposed the CXCR3⁺ PD-1⁺ cT_{FH} subset as the most related to *bona fide* GC-T_{FH} cells. Rather than being in conflict, these studies together indicate that cT_{FH} origin and phenotype may be intimately dependent on the nature of the antigenic trigger.

In healthy individuals, *Candida* species are harmless commensals that colonize skin and mucosal surfaces, but they can cause serious opportunistic fungal infections under certain conditions when the tissue homeostasis is disrupted. Mucosal homeostasis and host protection from *Candida* species is mainly ensured by Th17 cells, as demonstrated by the development of chronic mucocutaneous candidiasis in patients with inborn mutations in genes important for IL-17 immunity [37, 38], while antibodies are considered dispensable since patients with agammaglobulinaemia do not show increased susceptibility to *Candida* infections [39, 40]. Conversely, protection from influenza virus, a rapidly transmissible pathogen which cause acute respiratory infections, mainly relies on neutralizing antibodies acting sometimes together with CD4⁺ and CD8⁺ T cells [41, 42]. It is tempting to speculate that the distinct and highly differentiated antigen-specific cT_{FH} cell population that we measured in response to Flu, but not to *C.alb*, may be tightly linked to the magnitude of the antibody response and to the extent of antibody affinity maturation to Flu antigens, in line with the growing body of literature on cT_{FH} cells as correlates of the development of neutralizing antibodies [43, 44]. It is also conceivable that the magnitude of T_{FH} cell response and GC reactions might be different depending on the nature of the pathogen, hence resulting in the differentiation of a variable fraction of effector and memory cT_{FH} cells. Our study corroborates this notion by demonstrating that the lineage relationship between cT_{FH} and non-cT_{FH} cells is also largely dependent on the nature of the antigenic trigger.

The persistence of cT_{FH} cells as long-lived memory cells remains ill defined. In the context of Flu, we previously showed

that several T cell clones specific for an immunodominant epitope of influenza A hemagglutinin can be detected in the TCR V β repertoires of cT_{FH}, T_{CM} and T_{EM} analyzed several months later [33]. In the present study, we expanded this observation and showed that cT_{FH} cells specific for Flu antigens persisted as a stable repertoire over a time interval of 42 months similarly to Flu-specific T_{EM} cells, indicating clonal maintenance within the cT_{FH} subset as long-lived memory cells, in line with findings by Herati and colleagues [15]. These results are also consistent with a previous study showing that vaccinia virus-specific cT_{FH} cells were still detectable in vaccinated donors even 25 years after the smallpox eradication [45]. Moreover, our longitudinal study underlines the power of analysing the evolution of antigen-specific T cell repertoires by serial sampling. The frequent subset transitions we observed in the case of Flu-specific T_{CM} are consistent with a model of progressive differentiation of memory T cells that proposes T_{CM} as an intermediate stage [46, 47].

Overall, our findings suggest that differentiation and maintenance of cT_{FH} cells largely depend on the antigen context. Additional work will be needed to understand whether the heterogeneity in phenotype and longevity of cT_{FH} cells specific for different antigens is a correlate of a variable capacity of related *bona fide* GC T_{FH} cells to efficiently sustain antibody production and affinity maturation by B cells. Collectively, our study contributes to the understanding of the clonal relationship between antigen-specific cT_{FH} and non-cT_{FH} cells in the peripheral blood and provides insight on the heterogeneity, origin, and persistence of human cT_{FH} cells specific for different antigens.

Materials and methods

Cells and cell sorting

The study was approved by the Ethical committees of Cantone Ticino, Switzerland (Ref. 2018–02166/CE 3428). Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano. All blood donors provided written informed consent for participation in the study. Serial blood donations from a healthy donor who underwent annual Influenza vaccination were obtained after written informed consent. Human primary cell protocols were approved by the Federal Office of Public Health (no. A000197/2 to F.S.). Peripheral blood mononuclear cells (PBMCs) were isolated with Ficoll-Paque Plus (GE Healthcare). Monocytes and total CD4⁺ T cells were isolated by positive selection using anti-CD14 and anti-CD4 magnetic microbeads, respectively (Miltenyi Biotec). Total CD4⁺ cells obtained by positive selection were stained for chemokine receptors at 37°C and on ice for additional markers. Memory CD4⁺ T cells were sorted with a FACSria III (BD Biosciences) to over 98% purity by gating on CD4⁺CD8⁺CD14⁺CD16⁺CD19⁺CD25⁺CD56⁺CD45RA⁺ cells, and different memory CD4⁺ T cell subsets were isolated as follows: CXCR5⁺CCR6⁺, CXCR5⁺CCR6⁺, CXCR5⁺CCR6⁺, or CXCR5⁺PD-

1⁺, CXCR5⁺PD-1⁻, CXCR5⁻. In some experiments, after gating on CD4⁺CD8⁻CD14⁻CD16⁻CD19⁻CD25⁻CD56⁻CD45RA⁻ cells, cT_{FH} were sorted as total CXCR5⁺ cells, T_{CM} were sorted as CXCR5⁻CCR7⁺ cells, and T_{EM} were sorted as CXCR5⁻CCR7⁻ cells. The following fluorochrome-labeled mouse monoclonal antibodies were used for staining: CD4-APC (clone 13B8.2; cat. no. IM2468), CD45RA-Qdot 655 (clone MEM-56; cat. no. Q10069), CD45RA-FITC (clone ALB11; cat. no. A07786), CD8-PE-Cy5 (clone B9.11; cat. no. A07758), CD16-PE-Cy5 (clone 3G8; cat. no. A07767), CD56-PE/Cy5 (clone N901; cat. no. A07789), CD19-PE/Cy5 (clone J3-119; cat. no. A07771), CD25-PE-Cy5 (clone B1.49.9; cat. no. IM2646) from Beckman Coulter, CCR6-BV421 (clone 11A9; cat. no. 562515), PD-1-BV785 (clone EH12.2H7; cat. no. 329929), Human CXCR5 (BLR-1) MAb (Clone 51505; MAB190-100), Goat Anti-mouse IgG2b, human ads-BIOT (SB Cat. No. 1090-08), Streptavidin PE-CF594 (Cat. No. 562284), CD4-PE-Texas Red (clone S3.5; cat. no. MHCD0417) from ThermoFisher, PE/Cy7-Streptavidin (cat. no. 405206), ICOS-Pacific Blue (clone C398.4A; cat. no. 313521) from BioLegend.

Microbes and antigens

The influenza seasonal vaccines Influxac (Abbott, trivalent influenza vaccine) and Inflexal V (Crucell, trivalent influenza vaccine) were used at a concentration of 5 µg/ml for stimulation assays. *Candida albicans* SC5314 strain was processed in house. Briefly, *C. albicans* was cultured in YPD medium for 16 hours at 30°C, extensively washed in PBS, and heat inactivated at 65°C for 30 minutes. Ratio used for stimulation assays was three particles per monocyte. Lysate was prepared from mixed cultures of conidia and hyphae. Tetanus toxoid was obtained from Novartis Vaccines (Siena, Italy) and used at a concentration of 10 µg/ml for stimulation assays.

T cell stimulation

T cells were cultured in RPMI 1640 medium supplemented with 2 mM glutamine, 1% (vol/vol) nonessential amino acids, 1% (vol/vol) sodium pyruvate, penicillin (50 U/ml), streptomycin (50 µg/ml) (all from Invitrogen) and 5% human serum (Swiss Red Cross). Sorted memory CD4⁺ T cell subsets were labeled with carboxyfluorescein succinimidyl ester (CFSE) and cultured at a ratio of 2:1 with untreated or antigen-pulsed autologous monocytes. Before co-culture, autologous monocytes were irradiated with 45 Gy and pulsed for 3–5 hrs at 37°C with *C. albicans*, influenza vaccine, and tetanus toxoid. Antigen-stimulated memory T cell cultures were collected at day 6–7 and stained with antibodies to CD25-PE (clone M-A251; cat. no. 555432) from BD Biosciences and ICOS-Pacific Blue (clone C398.4A; cat. no. 313521 from BioLegend). T cell proliferation was determined by measuring the percentage of CFSE^{low} cells by flow cytometry followed by analysis with the FlowJo software. Proliferating memory T cells were FACS-sorted as CFSE^{low}CD25⁺ICOS⁺ and

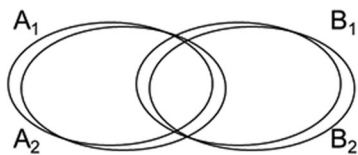
expanded in vitro in the presence of IL-2 (50 IU/ml) for subsequent experiments. Before sorting, cell culture supernatants of antigen-stimulated memory T cells were collected and preserved. Cytokine and chemokine concentration in the culture supernatants was assessed by Luminex bead-based multiplex assay according to manufacturer's instructions. In preliminary experiments, the frequency of antigen-specific T cells in the CFSE^{low} fraction was determined by performing single-cell cloning and analyzing the ability of the T cell clones to proliferate upon secondary antigenic stimulation and found to be between 90% and 100%.

TCR Vβ deep sequencing

Ex vivo-sorted memory CD4⁺ T cell subsets and CFSE^{low} fractions of antigen-stimulated memory CD4⁺ T cell cultures (2.5–5 × 10⁵ cells) were analyzed by deep sequencing. In brief, cells were centrifuged and washed in PBS, and genomic DNA was extracted from the pellet using QIAamp DNA Micro Kit (Qiagen), according to manufacturer's instructions. Genomic DNA quantity and purity were assessed through spectrophotometric analysis. Sequencing of TCR Vβ CDR3 was performed by Adaptive Biotechnologies using the ImmunoSEQ assay (<http://www.immunoseq.com>). In brief, following multiplex PCR reaction designed to target any CDR3 Vβ fragments, amplicons were sequenced using the Illumina HiSeq platform. Raw data consisting of all retrieved sequences of 87 nucleotides or corresponding amino acid sequences and containing the CDR3 region were exported and further processed. The assay was performed at deep level for ex vivo-sorted memory CD4⁺ T cell subsets (detection sensitivity, 1 cell in 200,000) and at survey level for CFSE^{low} antigen-reactive cultures (detection sensitivity, 1 cell in 40,000). Each clonotype was defined as a unique productively rearranged TCR Vβ nucleotide sequence; data processing was done using the productive frequency of templates provided by ImmunoSEQ Analyzer V3.0 (<http://www.immunoseq.com>). The overlap between pairs of TCR Vβ repertoires was quantified by calculating the following indexes. The percentage of shared clonotypes was calculated using the Jaccard index [$J = (A \cap B) / (A \cup B)$] as number of shared clonotypes between two subsets divided by the total number of clonotypes present in the same subsets, and normalized by 100. Cumulative frequencies of shared clonotypes were calculated as the average of the cumulative frequencies of the shared clonotypes in each of the two subsets.

To take into account the experimental variability due to the repertoire diversity and undersampling of lower abundant clonotypes, a normalized TCR overlap score based on technical replicates was calculated for each pair of antigen-specific TCR Vβ repertoires as follows. To measure the overlap between pairs of antigen-specific TCR Vβ repertoires, the Chao-Jaccard (CJ) similarity index was calculated using R package “fossil”. For each pairwise comparison of different T cell subsets, a normalization factor was defined as the lowest CJ value measured between the relating pairs of technical replicates. Lastly, CJ index calculated

between pairs of different antigen-specific TCR V β repertoires was divided by the normalization factor, to obtain a normalized TCR overlap score. In formula, if A_n and B_n are the TCR V β repertoires sequenced from two different T cell subsets A and B, and if A_1 and A_2 , and B_1 and B_2 are the corresponding technical replicates:



A normalized TCR overlap score between each pair of A_n and B_n TCR V β repertoires is calculated using Chao-Jaccard (CJ) similarity index, as follows:

$$\text{Normalized TCR overlap score} = \frac{\text{CJ}(A_n - B_n)}{\min[\text{CJ}(A_1 - A_2), \text{CJ}(B_1 - B_2)]}$$

Gene expression analysis

Antigen-reactive cells from each subset were sorted 24 h post-stimulation with antigens based on the upregulation of CD40L according to the protocol [26] and subjected to transcriptional analysis. Cell input from each sample was adjusted within the range from 6500 to 10,000 cells/sample. RNA was extracted and the expression levels for a total of 594 genes were measured by NanoString technology using nCounter Immunology Panel (Human_V2) (<https://nanosttring.com/products/ncounter-assays-panels/immunology/immunology-panel>) according to the manufacturer's instructions. In brief, 8 μ l of the hybridization Master Mix (proteinase K added with working concentration at 200 μ g/ml), including the Reporter CodeSet, was distributed in 12 tubes containing 4.5 μ l cell lysate from each sample. Subsequently, 2 μ l of the Capture ProbeSet and 0.5 μ l dH $_2$ O were added and, after mixing samples, the hybridization reaction was run at 65°C for 20 h. After the incubation, samples were loaded on the Nanostring cartridge lanes following the manufacturer's instructions. The assay was run on nCounter SPRINT Profiler device. Data were filtered by background thresholding and normalized by the nSolver analyzer software based on positive controls, negative controls, and housekeeping gene controls.

Statistical analysis

Statistical analysis was mainly performed using Prism software 8.0 (GraphPad). Significance was assigned at p -value < 0.05 unless stated otherwise. Specific tests are indicated in the figure legends for each comparison. Calculation of Chao-Jaccard similarity index and dendrogram analysis between TCR V β repertoires were performed using R software version 3.5.1. Network graphs showing the clonal relationship were performed using Cytoscape software.

Acknowledgments: The authors thank the blood donors for their participation in the study. This study was in part supported by the Swiss National Science Foundation (grants n. 310030L_182728 and 310030_189331 to F.S.) and the Institute for Research in Biomedicine are supported by the Helmut Horten Foundation.

Conflict of interest: The authors declare no commercial or financial conflict of interests.

Ethics Approval: The study was approved by the Ethical committees of Cantone Ticino, Switzerland (Ref. 2018–02166/CE 3428). Blood from healthy donors was obtained from the Swiss Blood Donation Center of Basel and Lugano. All blood donors provided written informed consent for participation in the study. Serial blood donations from a healthy donor who underwent annual Influenza vaccination were obtained after written informed consent. Human primary cell protocols were approved by the Federal Office of Public Health (no. A000197/2 to F.S.).

Author contributions: M.H., A.C., and F.S. were associated with conceptualization, methodology, and project administration. M.H., A.C., S.N., M.F., S.J., F.M., and D.J. performed Investigations. M.H., A.C., M.F., and F.S. performed visualization. F.S. acquired funding. A.C., A.L., and F.S. supervised the study. M.H. and A.C. wrote the original draft. A.C., A.L., and F.S. reviewed and edited the final manuscript.

Data availability statement: The data presented in this manuscript are included in the paper and in the Supplementary Information. TCR V β sequences have been deposited in the ImmuneAccess database (DOI: 10.21417/MH2022EJI).

Peer review: The peer review history for this article is available at <https://publons.com/publon/10.1002/eji.202250190>

References

- Breitfeld, D., Ohl, L., Kremmer, E., Ellwart, J., Sallusto, F., Lipp, M., Förster, R. et al., Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. *J. Exp. Med.* 2000;192(11):1545–52.
- Schaerli, P., Willmann, K., Lang, A. B., Lipp, M., Loetscher, P. and Moser, B., CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. *J. Exp. Med.* 2000;192(11):1553–62.
- Gitlin, A. D., Shulman, Z., Nussenzweig, M. C., Clonal selection in the germinal centre by regulated proliferation and hypermutation. *Nature.* 2014;509(7502):637–40.
- Victoria, G. D., Schwickert, T. A., Fooksman, D. R., Kamphorst, A. O., Meyer-Hermann, M., Dustin, M. L. and Nussenzweig, M. C., Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. *Cell.* 2010;143(4):592–605.

- 5 Fazilleau, N., Eisenbraun, M. D., Malherbe, L., Ebricht, J. N., Pogue-Caley, R. R., McHeyzer-Williams, L. J. and McHeyzer-Williams, M. G., Lymphoid reservoirs of antigen-specific memory T helper cells. *Nat. Immunol.* 2007;8(7):753–61.
- 6 Weber, J. P., Fuhrmann, F. and Hutloff, A., T-follicular helper cells survive as long-term memory cells. *Eur. J. Immunol.* 2012;42(8):1981–8.
- 7 Liu, X., Yan, X., Zhong, B., Nurieva, R. I., Wang, A., Wang, X., Martin-Orozco, N. et al., Bcl6 expression specifies the T follicular helper cell program in vivo. *J. Exp. Med.* 2012;209(10):1841–52, S1–24.
- 8 Hale, J. S., Youngblood, B., Latner, D. R., Mohammed, A. U., Ye, L., Akondy, R. S., Wu, T. et al., Distinct memory CD4⁺ T cells with commitment to T follicular helper- and T helper 1-cell lineages are generated after acute viral infection. *Immunity.* 2013;38(4):805–17.
- 9 Forster, R., Emrich, T., Kremmer, E. and Lipp, M., Expression of the G-protein-coupled receptor BLR1 defines mature, recirculating B cells and a subset of T-helper memory cells. *Blood.* 1994;84(3):830–40.
- 10 He, J., Tsai, L. M., Leong, Y. A., Hu, X., Ma, C. S., Chevalier, N., Sun, X. et al., Circulating precursor CCR7(lo)PD-1(hi) CXCR5(+) CD4(+) T cells indicate Tfh cell activity and promote antibody responses upon antigen reexposure. *Immunity.* 2013;39(4):770–81.
- 11 Locci, M., Havenar-Daughton, C., Landais, E., Wu, J., Kroenke, M. A., Arlehamn, C. L., Su, L. F. et al., Human circulating PD-1+CXCR3-CXCR5+ memory Tfh cells are highly functional and correlate with broadly neutralizing HIV antibody responses. *Immunity.* 2013;39(4):758–69.
- 12 Heit, A., Schmitz, F., Gerdt, S., Flach, B., Moore, M. S., Perkins, J. A., Robins, H. S. et al., Vaccination establishes clonal relatives of germinal center T cells in the blood of humans. *J. Exp. Med.* 2017;214(7):2139–52.
- 13 Benteibibel, S. E., Lopez, S., Obermoser, G., Schmitt, N., Mueller, C., Harrod, C., Flano, E. et al., Induction of ICOS+CXCR3+CXCR5+ TH cells correlates with antibody responses to influenza vaccination. *Sci. Transl. Med.* 2013;5(176):176ra32.
- 14 Herati, R. S., Reuter, M. A., Dolfi, D. V., Mansfield, K. D., Aung, H., Badwan, O. Z., Kurupati, R. K. et al., Circulating CXCR5+PD-1+ response predicts influenza vaccine antibody responses in young adults but not elderly adults. *J. Immunol.* 2014;193(7):3528–37.
- 15 Herati, R. S., Muselman, A., Vella, L., Bengsch, B., Parkhouse, K., Del Alcazar, D., Kotzin, J. et al., Successive annual influenza vaccination induces a recurrent oligoclonotypic memory response in circulating T follicular helper cells. *Sci Immunol.* 2017;2(8).
- 16 Brenna, E., Davydov, A. N., Ladell, K., McLaren, J. E., Bonaiuti, P., Metsger, M., Ramsden, J. D. et al., CD4(+) T Follicular Helper Cells in Human Tonsils and Blood Are Clonally Convergent but Divergent from Non-Tfh CD4(+) Cells. *Cell Rep.* 2020;30(1):137–52 e5.
- 17 Vella, L. A., Buggert, M., Manne, S., Herati, R. S., Sayin, I., Kuri-Cervantes, L., Bukh Brody, I. et al., T follicular helper cells in human efferent lymph retain lymphoid characteristics. *J. Clin. Invest.* 2019;129(8):3185–200.
- 18 Ma, C. S., Phan, T. G., Here, there and everywhere: T follicular helper cells on the move. *Immunology.* 2017;152(3):382–7.
- 19 Becattini, S., Latorre, D., Mele, F., Foglierini, M., De Gregorio, C., Cassotta, A. et al., T cell immunity. Functional heterogeneity of human memory CD4(+) T cell clones primed by pathogens or vaccines. *Science.* 2015;347(6220):400–6.
- 20 Acosta-Rodriguez, E. V., Rivino, L., Geginat, J., Jarrossay, D., Gattorno, M., Lanzavecchia, A., Sallusto, F. et al., Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* 2007;8(6):639–46.
- 21 Lindestam Arlehamn, C. S., Gerasimova, A., Mele, F., Henderson, R., Swann, J., Greenbaum, J. A., Kim, Y. et al., Memory T cells in latent Mycobacterium tuberculosis infection are directed against three antigenic islands and largely contained in a CXCR3+CCR6+ Th1 subset. *PLoS Pathog.* 2013;9(1):e1003130.
- 22 Cassotta, A., Goldstein, J. D., Durini, G., Jarrossay, D., Baggi Menozzi, F., Venditti, M., Russo, A. et al., Broadly reactive human CD4(+) T cells against Enterobacteriaceae are found in the naive repertoire and are clonally expanded in the memory repertoire. *Eur. J. Immunol.* 2021;51(3):648–61.
- 23 Viisanen, T., Ihanntola, E. L., Nanto-Salonen, K., Hyoty, H., Nurminen, N., Selvenius, J., Juutilainen, A. et al., Circulating CXCR5+PD-1+ICOS+ Follicular T Helper Cells Are Increased Close to the Diagnosis of Type 1 Diabetes in Children With Multiple Autoantibodies. *Diabetes.* 2017;66(2):437–47.
- 24 Obeng-Adjei, N., Portugal, S., Tran, T. M., Yazew, T. B., Skinner, J., Li, S., Jain, A. et al., Circulating Th1-Cell-type Tfh Cells that Exhibit Impaired B Cell Help Are Preferentially Activated during Acute Malaria in Children. *Cell Rep.* 2015;13(2):425–39.
- 25 Boswell, K. L., Paris, R., Boritz, E., Ambrozak, D., Yamamoto, T., Darko, S., Wloka, K. et al., Loss of circulating CD4 T cells with B cell helper function during chronic HIV infection. *PLoS Pathog.* 2014;10(1):e1003853.
- 26 Hegazy, A. N., West, N. R., Stubbington, M. J. T., Wendt, E., Suijker, K. I. M., Datsi, A., This, S. et al., Circulating and Tissue-Resident CD4(+) T Cells With Reactivity to Intestinal Microbiota Are Abundant in Healthy Individuals and Function Is Altered During Inflammation. *Gastroenterology.* 2017;153(5):1320–37 e16.
- 27 Yu, D., Vinuesa, C. G., The elusive identity of T follicular helper cells. *Trends Immunol.* 2010;31(10):377–83.
- 28 Havenar-Daughton, C., Lindqvist, M., Heit, A., Wu, J. E., Reiss, S. M., Kendrick, K., Bélanger, S. et al., CXCL13 is a plasma biomarker of germinal center activity. *Proc. Natl. Acad. Sci. U. S. A.* 2016;113(10):2702–7.
- 29 C. S. T Follicular, Helper Cell Biology: A Decade of Discovery and Diseases. *Immunity.* 2019;50(5):1132–48.
- 30 Ettinger, R., Sims, G. P., Fairhurst, A. M., Robbins, R., da Silva, Y. S., Spolski, R., Leonard, W. J. et al., IL-21 induces differentiation of human naive and memory B cells into antibody-secreting plasma cells. *J. Immunol.* 2005;175(12):7867–79.
- 31 Bryant, V. L., Ma, C. S., Avery, D. T., Li, Y., Good, K. L., Corcoran, L. M., de Waal Malefyt, R. et al., Cytokine-mediated regulation of human B cell differentiation into Ig-secreting cells: predominant role of IL-21 produced by CXCR5+ T follicular helper cells. *J. Immunol.* 2007;179(12):8180–90.
- 32 Avery, D. T., Bryant, V. L., Ma, C. S., de Waal Malefyt, R., Tangye, S. G., IL-21-induced isotype switching to IgG and IgA by human naive B cells is differentially regulated by IL-4. *J. Immunol.* 2008;181(3):1767–79.
- 33 Cassotta, A., Paparoditis, P., Geiger, R., Mettu, R. R., Landry, S. J., Donati, A., Benevento, M. et al., Deciphering and predicting CD4+ T cell immunodominance of influenza virus hemagglutinin. *J. Exp. Med.* 2020;217(10).
- 34 Brodie, T., Brenna, E., Sallusto, F., OMIP-018: chemokine receptor expression on human T helper cells. *Cytometry A.* 2013;83(6):530–2.
- 35 Lu, K. T., Kanno, Y., Cannons, J. L., Handon, R., Bible, P., Elkhouloun, A. G., Anderson, S. M. et al., Functional and epigenetic studies reveal multistep differentiation and plasticity of in vitro-generated and in vivo-derived follicular T helper cells. *Immunity.* 2011;35(4):622–32.
- 36 Zielinski, C. E., Corti, D., Mele, F., Pinto, D., Lanzavecchia, A., Sallusto, F., Dissecting the human immunologic memory for pathogens. *Immunol. Rev.* 2011;240(1):40–51.
- 37 Puel, A., Cypowyj, S., Bustamante, J., Wright, J. F., Liu, L., Lim, H. K. et al., Chronic mucocutaneous candidiasis in humans with inborn errors of interleukin-17 immunity. *Science.* 2011;332(6025):65–8.

- 38 Okada, S., Markle, J. G., Deenick, E. K., Mele, F., Averbuch, D., Lagos, M., Alzahrani, M. et al., IMMUNODEFICIENCIES. Impairment of immunity to *Candida* and *Mycobacterium* in humans with bi-allelic RORC mutations. *Science*. 2015;349(6248):606–13.
- 39 Richardson, J. P. and Moyes, D. L., Adaptive immune responses to *Candida albicans* infection. *Virulence*. 2015;6(4):327–37.
- 40 Netea, M. G., Joosten, L. A., van der Meer, J. W., Kullberg, B. J., van de Veerdonk, F. L., Immune defence against *Candida* fungal infections. *Nat. Rev. Immunol.* 2015;15(10):630–42.
- 41 Krammer, F., The human antibody response to influenza A virus infection and vaccination. *Nat. Rev. Immunol.* 2019;19(6):383–97.
- 42 Thomas, P. G., Keating, R., Hulse-Post, D. J. and Doherty, P. C., Cell-mediated protection in influenza infection. *Emerg. Infect. Dis.* 2006;12(1):48–54.
- 43 Koutsakos, M., Lee, W. S., Wheatley, A. K., Kent, S. J. and Juno, J. A., T follicular helper cells in the humoral immune response to SARS-CoV-2 infection and vaccination. *J Leukoc Biol.* 2021.
- 44 Wragg, K. M., Lee, W. S., Koutsakos, M., Tan, H. X., Amarasekera, T., Reynaldi, A., Gare, G. et al., Establishment and recall of SARS-CoV-2 spike epitope-specific CD4(+) T cell memory. *Nat. Immunol.* 2022.
- 45 Rivino, L., Messi, M., Jarrossay, D., Lanzavecchia, A., Sallusto, F. and Geginat, J., Chemokine receptor expression identifies Pre-T helper (Th)1, Pre-Th2, and nonpolarized cells among human CD4+ central memory T cells. *J. Exp. Med.* 2004;200(6):725–35.
- 46 Sallusto, F., Lenig, D., Forster, R., Lipp, M. and Lanzavecchia, A., Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. *Nature*. 1999;401(6754):708–12.
- 47 Lanzavecchia, A. and Sallusto, F., Progressive differentiation and selection of the fittest in the immune response. *Nat. Rev. Immunol.* 2002;2(12):982–7.

Full correspondence: Federica Sallusto, Institute of Microbiology, ETH Zurich, Zurich, Switzerland
e-mail: sallustf@ethz.ch

Received: 20/9/2022

Revised: 16/11/2022

Accepted: 7/12/2022

Accepted article online: 16/12/2022