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To cite this version:

Rafael Cubas, Julien Van Grevenynghe, Saintedym Wills, Lela Kardava, Brian H Santich, et al.. Reversible Reprogramming of Circulating Memory T Follicular Helper Cell Function during Chronic HIV Infection.. Journal of Immunology, Publisher : Baltimore : Williams Wilkins, e1950-. Latest Publisher : Bethesda, MD : American Association of Immunologists, 2015, 195 (12), pp.5625-36. <10.4049/jimmunol.1501524 >. <pasteur-01351053>

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_J Immunol_ 2015; 195:5625-5636; Prepublished online 6 November 2015;
doi: 10.4049/jimmunol.1501524
http://www.jimmunol.org/content/195/12/5625

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2015/11/06/jimmunol.1501524.DCSupplemental.html

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Reversible Reprogramming of Circulating Memory T Follicular Helper Cell Function during Chronic HIV Infection

Rafael Cubas,* Julien van Grevenynghe,† Saintedym Wills,‡ Lela Kardava,§ Brian H. Santich,¶ Clarisa M. Buckner,** Roshell Muir,† Virginie Tardif,** Carmen Nichols,* Francesco Procopio,** Zhong He,† Talibah Metcalf,** Khader Ghnime,†† Michela Loci,** Petronella Ancuta,†**, Jean-Pierre Routy,†‡‡ Lydie Trautmann,** Yuxing Li,* Adrian B. McDermott,** Rick A. Koup,** Constantinos Petros,‡‡ Steven A. Migueles,*** Mark Connors,*** Georgia D. Tomaras,‡ Susan Moir,** Shane Crotty,** and Elias K. Haddad*†‡‡‡§

Despite the overwhelming benefits of antiretroviral therapy (ART) in curtailing viral load in HIV-infected individuals, ART does not fully restore cellular and humoral immunity. HIV-infected individuals under ART show reduced responses to vaccination and infections and are unable to mount an effective antiviral immune response upon ART cessation. Many factors contribute to these defects, including persistent inflammation, especially in lymphoid tissues, where T follicular helper (Tfh) cells instruct and help B cells launch an effective humoral immune response. In this study we investigated the phenotype and function of circulating memory Tfh cells as a surrogate of Tfh cells in lymph nodes and found significant impairment of this cell population in chronically HIV-infected individuals, leading to reduced B cell responses. We further show that these aberrant memory Tfh cells exhibit an IL-2–responsive gene signature and are more polarized toward a Th1 phenotype. Treatment of functional memory Tfh cells with IL-2 was able to recapitulate the detrimental reprogramming. Importantly, this defect was reversible, as interfering with the IL-2 signaling pathway helped reverse the abnormal differentiation and improved Ab responses. Thus, reversible reprogramming of memory Tfh cells in HIV-infected individuals could be used to enhance Ab responses. Altered microenvironmental conditions in lymphoid tissues leading to altered Tfh cell differentiation could provide one explanation for the poor responsiveness of HIV-infected individuals to new Ags. This explanation has important implications for the development of therapeutic interventions to enhance HIV- and vaccine-mediated Ab responses in patients under ART.

T follicular helper (Tfh) cells are a specialized subset of CD4+ T cells that provide help to B cells in germinal centers (GCs) and instruct B cell differentiation into affinity-matured, long-lived memory B cells and plasma cells (1). Tfh cells deliver critical signals to GC B cells via costimulatory molecules and lymphokine secretion and induce somatic hypermutation.

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Received for publication July 7, 2015. Accepted for publication October 6, 2015.

This work was supported by National Institutes of Health Grant R01AI106482-01 to E.K.H.; the Mathilde Krim Fellowship from the American Foundation for AIDS Research (Grant 108671555/RKVA) to (R.C.); and in part by Canadian Institutes of Health Research Grant 103230, the Canadian Institutes of Health Research Canadian Trials Network/HIV Trial Network (CTN 247), the Fonds de la Recherche en Santé du Québec-Maladies Infectieuses, and the Duke Center for AIDS Research Immunology Core (National Institutes of Health Grant AI064518).

Y.L. was funded by National Institutes of Health/National Institute of Allergy and Infectious Diseases Grant R01AI102766 and National Institute of Allergy and Infectious Diseases Development Grant P30AI68514, the Center for AIDS Research, and the University of California, San Diego. J.-P.R. holds the Louis Lowenstein Chair in Hematology-Oncology, McGill University.

R.C. designed the study, developed and performed experiments, interpreted the data, generated figures, and prepared the manuscript; J.v.G. and Z.H. collaborated on the coculture assay development and planning; S.W. performed and analyzed the HIV-1 multiplex binding assays; L.K. performed and analyzed the evaluation of Ag-specific B cells by flow cytometry; B.H.S. and C.M.B. helped with B cell assays; F.P., C.N., and R.M. helped with the BioMark assays and primer libraries; R.M. and V.T. helped with some of the coculture assays and ELISAs; T.M. assisted in the processing of blood samples; K.G. helped with statistical analysis; M.L. provided intellectual input; P.A. and J.-P.R. provided valuable samples and intellectual input and discussion; L.T. provided valuable discussion; Y.L. provided the HIV YU2 gp120 probe and A.B.M. provided the H11N1 hemagglutinin probe as well as intellectual input for the detection of Ag-specific B cells by flow cytometry; R.A.K. and C.P. provided intellectual input and discussion; S.A.M. and M.C. provided valuable samples and discussion; G.D.T. provided valuable input and discussion and helped to design and analyze the HIV-1 multiplex binding assays; S.M. provided valuable intellectual input and discussion and helped to design and analyze the evaluation of Ag-specific B cells by flow cytometry; S.C. provided valuable intellectual input and discussion and helped design experiments; E.K.H. conceived and directed the research, designed experiments, analyzed data, and helped write the manuscript.

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The online version of this article contains supplemental material.

Abbreviations used in this article: ART, antiretroviral therapy; ASC, Ab-secreting cell; CA, chronic atypical; cTfh, circulating Tfh; EC, elite controller; GC, germinal center; HC, healthy control; LN, lymph node; MFI, mean fluorescence intensity; NIH, National Institutes of Health; RT, room temperature; SEB, staphylococcal enterotoxin B; Tfh, T follicular helper.

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on Ag-specific B cells (1, 2). The differentiation of Tfh cells involves multiple signals that lead to the activation of various transcription factors, with the core programming being largely mediated by Bcl6, Maf, STAT3, and STAT4 transcription factors (3). IL-2 is a potent inhibitor of Tfh cell differentiation, as it interferes with Bcl6 and CXCR5 expression (4–6) and is dose limiting for Th1 versus Tfh cell differentiation (4). In humans, administration of IL-2 in vivo did not yield any clinical benefits to HIV-infected subjects. However, PBMCs obtained from IL-2–treated patients showed increased in CD25 expression and production of inflammatory cytokines (7, 8).

During HIV infection, vast cytokine imbalances (9) in lymphoid tissues can persist despite antiretroviral therapy (ART), affecting both T cell and B cell homeostasis (10). It has recently been shown that HIV continues to undergo low-level replication in lymphoid tissues, maintaining a state of immune activation in individuals undergoing ART. In addition, B cell follicles appear to represent protected niches where viral replication can persist despite the presence of strong antiviral CD8+ T cell responses (11). It is therefore conceivable that persistent inflammatory signals could skew the differentiation profile of Tfh cells, leading to an altered polarization and adoption of cytokine signaling programs, which could affect their ability to provide adequate B cell help. The lymph node (LN) microenvironment is likely responsible for inducing major changes on Tfh cell function. As evidence for this, we have previously shown that despite an increase in the frequency of Tfh cells, their function is impaired in LNs from HIV-infected individuals (12).

Recently, a population of blood circulating Tfh (cTfh) cells has been described that represents a circulating memory counterpart of LN-resident Tfh cells (13–15). If cTfh cells arise from Tfh cell precursors in LNs, changes in the LN microenvironment could imprint particular effector fates or functional skewing during their differentiation into memory cells. With the difficulty of obtaining LN samples, investigating the functional status of memory Tfh cells in peripheral blood could provide a glimpse into the microenvironmental changes that took place in LNs during the differentiation of these cells into memory counterparts. With the recent demonstration that HIV continues to undergo low-level replication in lymphoid tissues maintaining a state of immune activation despite ART (16), it is conceivable that Tfh cells encounter environmental signals, which could affect their differentiation and phenotype when transitioning into memory Tfh cells.

In this article, we show that memory Tfh cells from peripheral blood of chronic aviremic (CA) individuals are functionally impaired in their ability to provide adequate B cell help when compared with those from elite controllers (ECs). This impairment stems in part from increased IL-2 signaling, which can affect the differentiation and function of memory Tfh cells. We further demonstrate that memory Tfh cells in CA individuals are more polarized toward a Th1 phenotype with increased levels of Tbet expression; increased secretion of IFN-γ; and reduced expression of Bcl6, CXCR5, and IL-21. Importantly, memory Tfh cells from CA subjects are reprogrammable and still show plasticity, as their function can be restored at least partly by interfering with IL-2 signaling.

### Materials and Methods

#### Human samples

All procedures were approved by the Institutional Review Boards at the relevant institutions, and all participants provided signed informed consent. Frozen PBMCs were obtained from CA individuals and ECs who were recruited from the Clinical Research Center, National Institutes of Health (NIH). Patient information can be found in Tables I and II. PBMCs from CA individuals and ECs were also obtained from patients who participated in the blood banking study from the Chronic Viral Illness Service at McGill University Health Centre. Informed consent was signed by each participant, and the study was approved by the Research Ethics Board of

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**Table I. Patient information on the CA-treated individuals used in various assays**

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<th>CD8+ T Cell Count (cells/mm3)</th>
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HIV-infected CA individuals were assessed for CD4 and CD8 counts as well as virus load. NA, not available.
the Research Institute of McGill University Health Centre. Blood samples from healthy donors were obtained from Martin Memorial Health Systems. PBMCs were isolated from blood by density-gradient centrifugation using Ficoll-Paque PLUS (GE Healthcare). Cells were subsequently frozen and used for the relevant assays. Fresh human tonsils were obtained from tonsillectomies performed at Martin Memorial Health Systems. Tonsils were processed as previously described (10).

**Abs**

The following fluorochrome-conjugated Abs were used: anti–human CD3 (clone UCHT1, 1:200), anti–human CD4 (clone RPA-T4, 1:200), anti–human CD19 (clone HIB19, 1:100), anti–human CD27 (clone O323, 1:100), anti–human CD38 (clone HIT2, 1:100), anti–human CD95 (clone DX2, 1:100), anti–human ICOS (clone C398.4A, 1:100), anti–human CD40L (clone 24-31, 1:10), anti–human PD-1 (clone EH12.2H7, 1:100), anti–human CXCR3 (clone G025H7, 1:200), anti–human CTLA4 (clone 1H10, 1:100), anti–human ICOS (clone C398.4A, 1:100), anti–human CD38 (clone HIT2, 1:100), anti–human CD95 (clone UCHT1, 1:200), anti–human CD4 (clone RPA-T4, 1:200), anti–human IL-7R (clone A019D5, 1:100) were all from BioLegend. Anti–human CXCR5 (clone RF8 B2, 1:200), anti–human Bcl-6 (clone K112-91, 1:20), anti–human IgD (clone IA6-2, 1:50), anti–human Ki-67 (MOPC-21, 1:10), and anti–human CD21 (clone B-Ly4, 1:100) were from BD Biosciences, and anti–human CD45RA (clone 2H4LDH11LDB9, 1:200) was from Beckman Coulter. LIVE/DEAD Fixable Dead Cell Stain from Invitrogen was used to gate on live cells as well as Annexin V from BD Biosciences.

**Coculture assays**

The cTfh cells defined as (CD3+CD4+CD45RA-CD25+CXCR5+CXCR3+) or the non-cTfh cells defined as (CD3+CD4+CD45RA+CXCR5+CXCR3-) were sorted and placed in coculture with memory B cells (CD3+CD19+CD27+) at equal numbers (1 × 10^6 cells each) in the presence of 100 ng/ml staphylococcal enterotoxin B (SEB) (Toxin Technology) in complete RPMI 1640 medium (Life Technologies). Supernatants and cells were harvested at 3, 5, or 7 d for subsequent analysis. SEB was used to add at the onset of the cocultures to mimic the interaction between T cells and B cells in a polyclonal setting (13). The presence of SEB was required to induce T cell activation and subsequent interaction between T cells and B cells in a polyclonal setting (13). The following fluorochrome-conjugated Abs were used: anti–human CD3 (clone UCHT1, 1:200), anti–human CD45RA (clone 2H4LDH11LDB9, 1:200) was from BD Biosciences, and anti–human CD45RA (clone 2H4LDH11LDB9, 1:200) was from BD Biosciences.

**Table II. Patient information on the ECs used in various assays**

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</tbody>
</table>

HIV-infected ECs were assessed for CD4 and CD8 counts as well as virus load. NA, not available.

**ELISA**

ELISAs were performed as previously described (10). In brief, for analysis of total IgG output from the cocultures, 96-well Immunol 2HB plates (Thermo Scientific) were coated overnight at 4°C with monoclonal anti-human IgG (Mabtech; clone MT91/145) at a concentration of 1 μg ml^-1 in PBS. The following day, plates were washed five times with PBS + 0.05% Tween 20. Plates were subsequently blocked for 1 h with PBS + 10% FBS at room temperature (RT). Plates were subsequently washed and the culture supernatants added at different dilutions for 1 h at RT. After washing, the plates were incubated with 1 μg ml^-1 of anti-human IgG–biotin (Mabtech, clone MT78/145) for 1 h at RT. The plates were then washed as above and incubated with streptavidin-HRP (Mabtech) for 45 min at RT. Plates were thoroughly washed, and 100 μl tetramethylbenzidine substrate (Sigma-Aldrich) was added until the appearance of color. The enzymatic reaction was stopped by the addition of 50 μl 1 M H₃PO₄. The OD (450 nM) was then measured using a SpectraMax plus 384-plate reader (Molecular Devices).

To detect Env-specific Ab responses, 96-well, high-binding, half-area ELISA plates (Greiner Bio-One) were coated overnight at 4°C with 1 μg ml^-1 recombinant HIV-1 envelope protein (ProSpec) in PBS. The following day, plates were washed five times with PBS + 0.05% Tween 20 and blocked for 1 h with PBS + 10% FBS at RT. Plates were subsequently washed and the culture supernatants added at different dilutions for 2 h at RT. For the standard curve, human HIV Ig was used. This reagent was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH (cat. no. 3957) from Dr. Luiz Barbosa, National Heart Lung and Blood Institute. The plates were then washed and incubated with 1 μg ml^-1 anti–human IgG–biotin (Mabtech; clone MT78/145) for 45 min at RT. Plates were then thoroughly washed, and 50 μl tetramethylbenzidine substrate (Sigma-Aldrich) was added until the appearance of color. The enzymatic reaction was stopped by the addition of 25 μl 1 M H₃PO₄. The OD (450 nM) was then measured using a SpectraMax plus 384-plate reader (Molecular Devices).

**BioMark**

Assays (Primers and Probes) were designed using the Roche Universal Probe Library Assay Design Center (www.universalprobelibrary.com) and were designed to detect multiple transcripts, without respect to isoform prevalence. Cells were sorted directly into PCR plates and immediately frozen. RNA extraction and PCR amplifications were done according to manufacturer recommendations and as we have previously published (17).

**Evaluation of Ag-specific B cells by flow cytometry**

Mature B cells were stained with Abs against B cell markers, as well as fluorochrome-conjugated probes derived from HIV YU2 gp140 and influenza H1N1 HA, as previously described (18). HIV-specific memory B cells were identified using a trimeric HIV envelope gp140 probe. This probe contains a site-specific biotinylation motif Avi tag at the C termini that can be fluorescently labeled. The HIV-gp140 probe was conjugated with streptavidin-allophycocyanin (Invitrogen). FACS analyses were
performed using a FACSCanto II flow cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

**Statistical analysis**

All data were presented as means ± SD. Comparison between cohorts (healthy control [HC], EC, and CA) were performed using a two-tailed Mann–Whitney U test. This analysis is optimal for clinical cohorts, as it does not assume normal Gaussian distribution of the data. For statistical analyses within the same group using autologous or allogeneic cocultures (substitution of one cell type), we used paired, nonparametric Wilcoxon matched-pairs signed rank tests. For statistical analyses within the same group (i.e., comparison between Ab levels in cocultures from ECs with or without supplementation of IL-2 or IFN-γ) two-tailed paired t tests were used. Prism 5.0 (GraphPad) was used to process all the statistical analyses.

**Results**

**Memory Tfh cells from CA individuals are functionally impaired**

First, we characterized the frequency and phenotype of cTfh cells in peripheral blood from a cohort of HC, EC, and CA subjects (Tables I and II). Memory Tfh cells are Ag experienced (CD45RA−), have mostly a central-memory phenotype (CCR7+CD27+), and have stable expression of CXCR5. These cells have also been characterized as lacking the chemokine receptor CXCR3 (13, 14, 19). Unlike their GC counterparts, memory Tfh cells express high levels of IL-7Rα, critical for long-term survival, and have a resting phenotype (CD69−, Ki-67−). The majority of memory Tfh cells also lack CD38 and ICOS expression (Supplemental Fig. 1A–E), but a proportion of them express PD-1 (Supplemental Fig. 1F), as previously shown (14). When comparing the three patient groups, we did not observe any significant difference in the frequency and absolute number of cTfh cells (Supplemental Fig. 1G). In addition, we did not detect any significant difference in the expression of costimulatory (ICOS, CD40L), inhibitory (PD-1), or activation (CD38) markers following stimulation with anti-CD3/CD28 (Supplemental Fig. 1H). These results suggest that under anti-CD3/CD28 stimulation, at least for the markers studied, there are no apparent differences in the phenotype of memory Tfh cells between HC, EC, and CA subjects. However, the functional aspects of cTfh cells are better investigated in the context of B cell interaction

FIGURE 1. cTfh cells from CA individuals are functionally impaired in their ability to provide adequate B cell help. (A) PBMCs from HC subjects were sorted into cTfh cells (CD3+CD4+CD45RA−CXCR5+CXCR3−), non-cTfh cells (CD3+CD4+CD45RA−CXCR5−CXCR3+ and CD3+CD4+CD45RA−CXCR5−), and CD27+ memory B cells and placed in coculture for 7 d in the presence of SEB. Shown are the total levels of IgG in the coculture supernatants after 3, 5, and 7 d, as measured by ELISA. n = 4–12. (B and C) PBMCs from HC, EC, and CA subjects were also sorted into cTfh cells and memory B cells and placed in coculture for 7 d in the presence of SEB. (B) Total levels of IgG after 7 d. (C) Absolute number of live B cells after 7 d. n = 16–24. (D–H) Alternatively, either cTfh cells or memory B cells from a sorted pool of HCs were used to substitute cTfh cells or memory B cells in cocultures from EC or CA subjects. (D) Total levels of IgG after replacing cTfh cells from CA subjects with cTfh cells from HCs. (E) Total levels of IgG after replacing cTfh cells from ECs with cTfh cells from HCs. (F) Comparison of total IgG levels in cocultures from ECs with CA subjects with substituted cTfh cells from HCs. (G) Total IgG levels in cocultures after replacing cTfh cells from ECs and CA subjects with cTfh cells from HCs. (H) Total IgG levels in cocultures after replacing memory B cells from ECs and CA subjects with memory B cells from HCs. Bars represent mean ± SD. (D–H) n = 8. Each dot represents the average of samples done in triplicate.
and crosstalk. To accomplish this, we performed coculture assays with sorted memory Tfh cells (defined as CXCR5+CXCR3+CD4+ memory T cells) and sorted memory B cells (defined as CD3-CD19+CD27+). As a control, we also cocultured non-Tfh cells (defined as CXCR5+CXCR3+ or CXCR5-CD4+ memory T cells) with memory B cells.

Similar to their GC counterparts, cTfh cells are specialized in providing B cell help. This characteristic was evidenced by both Ab output (Fig. 1A) and the absolute number of proliferating B cells after 7 d in coculture (Supplemental Fig. 2A). CXCR3+ cells did not provide adequate B cell help, despite having a similar proliferative capacity to cTfh cells (Fig. 1A, Supplemental Fig. 2B), corroborating previous findings (13). In addition, activation of Tfh cells (using SEB) was required for their adequate function (Supplemental Fig. 2C). The absolute number of live B cells in the coculture was positively correlated with the total level of IgG (data not shown), demonstrating a relationship between cTfh-mediated B cell help and Ab production. From these results, it is evident that cTfh cells defined as CXCR5+CXCR3-CD4+ memory T cells provide optimal help to memory B cells.

Using this coculture assay on 17 HC, 16 EC, and 24 CA individuals, we found that cocultures from CA subjects showed significantly lower levels of total IgG Ab output when compared with cocultures from ECs and HCs (Fig. 1B, p < 0.001). The differences in IgG levels were not due to a decrease in the absolute number of live B cells (Fig. 1C) or cTfh cells, as the absolute number of these cells was comparable between EC and CA subjects (Supplemental Fig. 2D). This finding suggests that the blunted IgG Ab levels could be due to defective cTfh/B cell interaction, which could influence memory B cell differentiation into IgG-secreting cells. To investigate this possibility further, we determined if we could restore Ab production by exchanging cTfh cells from CA subjects with cTfh cells from HC subjects, while maintaining the same memory B cells from CA individuals. Our results showed that replacing cTfh cells from CA subjects with cTfh cells from HC subjects significantly increased total IgG Ab output in cocultures with memory B cells from CA subjects (Fig. 1D, p < 0.01). The restoration in Ab production was not due to an allogeneic effect, as memory B cells cocultured with allogeneic cTfh cells from HC subjects in the absence of stimulation (SEB) did not secrete detectable levels of IgG and showed poor viability (Supplemental Fig. 2G). Furthermore, in cocultures with memory B cells from ECs, replacing cTfh cells from ECs with cTfh cells from HCs did not enhance Ab production (Fig. 1E). In addition, the mean fluorescence intensity (MFI) of CD27, a
marker highly expressed on Ab-secreting cells (ASCs), was significantly reduced in cocultures from CA subjects but restored when these cells were cultured with cTfh cells from HCs (Supplemental Fig. 2E, 2F, p < 0.001). This observation suggests that memory B cells from CA subjects have a reduced capacity to differentiate into ASCs in the presence of impaired autologous cTfh cells.

For CA individuals, replacing autologous cTfh cells with those from HCs restored Ab production to the levels observed in ECs (Fig. 1F). This observation indicated that the memory B cell compartment in CA subjects had a similar intrinsic capacity to differentiate into ASCs when compared with ECs. This finding was further corroborated by coculturing memory B cells from CAs and ECs with cTfh cells from HCs, showing that memory B cells from both EC and CA subjects produced similar quantities of IgG (Fig. 1G).

The impairment in cTfh cell function in CA individuals became more apparent when we cocultured memory B cells from HCs with cTfh cells from either ECs or CA subjects. The results indicated that total IgG Ab output with cTfh cells from CA individuals was significantly decreased when compared with cocultures with cTfh cells from ECs (Fig. 1H, p < 0.02). Together, these results suggest that the reduced Ab production observed in cocultures from CA subjects was primarily due to dysfunctional cTfh cells rather than intrinsic memory B cell defects.

**Memory Tfh cells from CA subjects show increased IL-2 signaling**

To continue characterizing the cTfh cell defect in CA subjects, we sorted cTfh cells after 7 d in coculture with memory B cells for Fluidigm BioMark analysis and compared their transcriptional gene fold change to sorted cTfh cells on day 0. We observed that some cTfh cells from cocultures of CA subjects showed a tendency toward increased IL-2Rα expression and lower levels of BCL6, CXCR5 and IL21, canonical Tfh related genes essential for Tfh cell differentiation, trafficking, and B cell help when compared with cTfh cells from ECs (Fig. 2A). Even though the number of samples used in this assay was limited, the results suggested possible trends in the expression of some of these genes and made us pursue these differences further at the protein levels. Thus, we investigated expression of the high-affinity IL-2 receptor (IL-2Rα/CD25) at the protein level in cocultures from CA individuals and ECs. We observed a significant increase (p < 0.02) in the frequency of CD25+ cTfh cells in cocultures from CA individuals, whereas the expression levels of CXCR5 were significantly reduced (p < 0.001) (Fig. 2B, 2C). The increase in CD25+ cTfh cells was not due to signals coming from the memory B cells, as cocultures of memory B cells from CA subjects with cTfh cells from HCs did not result in an increase of CD25 expression on the cTfh cells (Fig. 2B). This finding suggests that the increase in CD25 expression was due to intrinsic properties in the cTfh cell compartment from CA individuals. In addition, we observed significant negative correlations between CD25 expression on cTfh cells and their expression level of CXCR5 and IgG Ab output in the cocultures (Fig. 2D, 2E, p < 0.001 and p < 0.01, respectively). On the contrary, we observed a significant positive correlation between CXCR5 MFI on cTfh cells and total IgG Ab levels (Fig. 2F, p < 0.0001). Together, these results imply that cTfh cells from CA subjects can display increased IL-2 signaling, which could be partly responsible for reprogramming these cells and altering their polarization and capacity to provide B cell help.

The expression of CD25 is regulated by TCR stimulation but can be sustained by IL-2 in a positive feedback loop involving STAT5 (20, 21). Indeed, we observed significantly higher levels of IL-2 in cocultures from CA subjects (Fig. 3A, p < 0.01), and these levels were significantly reduced when exchanging cTfh cells from CA subjects with those from HCs. In addition, exchanging the memory B cells did not affect the levels of IL-2 in cocultures from CA subjects, indicating that the increased levels of IL-2 were coming from the cTfh cells (Fig. 3A). Furthermore, we observed a significant positive correlation between the levels of IL-2 and the expression of CD25 on cTfh cells (Fig. 3B, p < 0.03). The increase in IL-2 was also negatively correlated with total IgG Ab output (Fig. 3C, p < 0.02). These results suggest that the reprogramming of cTfh cells during chronic HIV infection could be partly the result of increased IL-2 signaling on these cells. A similar increase in the levels of IL-2 was also observed in coculture of non-cTfh cells (CXCR5+CXCR3+) and memory B cells when compared with cocultures from cTfh cells in HCs (Fig. 3D). This observation suggests that the cTfh cells in CA subjects are skewed toward a non-cTfh phenotype and increased IL-2 production.
Memory Tfh cells from CA individuals are more polarized toward a Th1 phenotype

Triggering of IL-2R could activate downstream signals that could interfere with the STAT3-Bcl6 axis, skewing the polarization of memory Tfh cells. As evidence for this, we observed significantly higher levels ($p < 0.001$) of the characteristic Th1 cytokine IFN-$\gamma$ (22, 23) in cocultures from CA individuals. Similar to what we observed for IL-2, the levels of IFN-$\gamma$ were also significantly reduced when cTfh cells from HCs were used in the cocultures ($p < 0.01$). We also observed a significant negative correlation between IFN-$\gamma$ and total IgG levels ($p < 0.001$). Moreover, we observed a significant increase in TNF-$\alpha$, another characteristic Th1 cytokine in cocultures from CA subjects ($p < 0.001$). As with IFN-$\gamma$, the levels of TNF-$\alpha$ were significantly reduced by exchanging with cTfh cells from HCs ($p < 0.01$). Furthermore, a significant negative correlation was found between the levels of total IgG and TNF-$\alpha$ ($p < 0.002$). IL-2 showed a positive correlation with both IFN-$\gamma$ ($p < 0.01$) and TNF-$\alpha$ ($p < 0.0001$), and TNF-$\alpha$ showed a positive correlation ($p < 0.0002$) with IFN-$\gamma$ (Fig. 4E-G). In addition, we also observed a similar increase in the levels of IFN-$\gamma$ and TNF-$\alpha$ when coculturing non-cTfh cells (CXCR5$^+$CXCR3$^+$) with memory B cells in samples from HCs (Fig. 4H), suggesting that cTfh cells in CA subjects appear to be more polarized toward a Th1 phenotype.

IL-2 can reprogram memory Tfh cell function

We then supplemented memory Tfh/B cell cocultures from ECs with IL-2 or IFN-$\gamma$ to determine whether we could recapitulate the functional defects observed with cTfh cells from CA subjects. As shown in Fig. 5A and 5B, IL-2 supplementation led to a significant decrease in total IgG Ab levels ($p < 0.03$), whereas IFN-$\gamma$ reduced the levels of total IgG, but not to the same extent as observed with IL-2 supplementation. The decrease in Ab output in the presence of IL-2 was not due to differences in the absolute number of cTfh cells, as their numbers were similar (in the case of IFN-$\gamma$) or reduced (in the case of IL-2) when compared with the controls (Fig. 5C). Supplementation with IL-2 also increased the expression of CD25 ($p < 0.01$) on cTfh cells while decreasing CXCR5 expression ($p < 0.001$) (Fig. 5D, 5E). In addition, CD27 MFI was significantly decreased on B cells, suggesting reduced differentiation into ASCs (Fig. 5F, $p < 0.01$). Importantly, IL-2 supplementation significantly increased the levels of the Th1 transcription factor Tbet ($p < 0.01$) on cTfh cells as well as the transcription factor Tbet (Fig. 5G, $p < 0.01$) on cTfh cells.
Blimp1, which antagonizes Bcl6 expression and Tfh cell differentiation (Fig. 5H, \( p < 0.01 \)) (24). We also observed a positive correlation between Tbet and Blimp1 expression on these cells (Fig. 5I, \( p < 0.02 \)). Samples supplemented with IL-2 also showed increased levels of IFN-\( \gamma \) and TNF-\( \alpha \) production (Supplemental Fig. 3A, 3B). However, samples supplemented with IFN-\( \gamma \) did not show an increase in IL-2 production or TNF-\( \alpha \) levels (Supplemental Fig. 3C, 3D), suggesting that increased IL-2 signaling might be an important driver or maintainer of memory Tfh cell polarization toward a Th1-like phenotype in CA subjects.

To determine whether the aberrant Th1 polarization on cTfh cells from CA subjects could be reversed, we interfered with IL-2 signaling in the coculture. This was achieved by adding a titrated amount of αIL-2 Ab to the cocultures to reduce the heightened levels of IL-2 without completely blocking signaling through CD25, which is required for both cTfh and B cell survival (data not shown). Reducing the levels of IL-2 in cocultures from CA individuals led to an increase in total IgG Ab output (Fig. 6A, \( p < 0.01 \)) as well as decreased CD25 expression (Fig. 6B, \( p < 0.01 \)) and increased CXCR5 levels on cTfh cells (Fig. 6C, \( p < 0.01 \)). In addition, we observed a significant decrease in the expression of the transcription factor Tbet, which plays a major role in driving Th1 differentiation (Fig. 6D, \( p < 0.01 \)) as well as Blimp1 (Fig. 6E, \( p < 0.02 \)), which can block Bcl6 expression and Tfh cell differentiation (24). Furthermore, increased Tbet expression was significantly correlated (Fig. 6F, \( p < 0.01 \)) with decreased IgG levels as well as decreased CXCR5 expression on cTfh cells (Fig. 6G, \( p < 0.0001 \)). On the contrary, increased expression of Tbet on cTfh cells was significantly and positively correlated with increased expression levels of CD25 (Fig. 6H, \( p < 0.0001 \)) and Blimp1 (Fig. 6I, \( p < 0.0001 \)). Thus, the apparent Th1-like programming of cTfh cells in CA subjects can be partly reversed by dampening IL-2 signaling, leading to a more functional cTfh cell phenotype and an improved capacity to provide B cell help.

HIV-specific Ab responses in CA subjects can be enhanced by adequate cTfh cell help

We next determined whether we could enhance the production of HIV-specific Abs by providing adequate B cell help. We first determined whether HIV-specific memory B cells were present in our cohort of CA subjects, as HIV-infected individuals under ART show a continuous loss of HIV-specific memory B cells (17, 25–28). We used a fluorochrome-labeled gp140 probe to detect Ag-specific memory B cells by flow cytometry. We observed that, compared with ECs, there was a significant reduction in the frequency of
gp140-specific memory B cells in CA individuals (Fig. 7A, \(p < 0.001\)). We also observed a reduction in influenza-specific memory B cells, although the difference did not reach statistical significance (data not shown). We then measured HIV-specific Ab levels in cocultures from EC and CA subjects. As expected, we found significantly lower levels of HIV-specific Abs in cocultures from CA subjects, and the majority of these individuals did not have measurable HIV-specific Abs (Fig. 7B, \(p < 0.00001\)). This decrease in HIV-specific responses from CA subjects was evident for Abs against gp140 (\(p < 0.001\)) and gp41 (\(p < 0.001\)) and the predominant IgG subclasses for anti-HIV responses: IgG1 (\(p < 0.01\)) and IgG3 (\(p < 0.001\)) (Supplemental Fig. 4). Reduced production of IL-21 by cTfh cells from CA subjects, as suggested by our BioMark data, could partly explain the reduced levels of HIV-specific IgG and IgG3 (29). Importantly, when we supplemented cocultures from ECs with IL-2 or IFN-\(\gamma\), we observed a significant reduction in HIV-specific Ab levels (\(p < 0.001\) and \(p < 0.02\), respectively) that reached levels similar to those observed in cocultures from CA subjects (Fig. 7C, 7D). However, when cTfh cells from CA individuals were replaced in the cocultures for cTfh cells from HCs, the level of Env-specific IgG was increased (Fig. 7E, \(p < 0.01\)). We also observed a significant increase in the level of Env-specific IgG when cocultures from CA subjects were supplemented with recombinant IL-21 (Fig. 7F, \(p < 0.03\)). Thus, in the presence of functional cTfh cell help, Env-specific IgG levels are enhanced in cocultures from CA subjects.

**Discussion**

In this study, we have shown that cTfh cells from CA individuals are functionally impaired in their ability to provide adequate B cell help when compared with memory Tfh cells from ECs and HCs. Memory Tfh cells from CA subjects showed increased IL-2 signaling as well as impaired differentiation and function. In accordance, studies in mice have shown a negative role of IL-2 signaling in mediating Tfh cell differentiation (4–6). Increased autocrine IL-2 levels could be leading to heightened STAT5 signaling, which has been shown to repress Bcl6 expression by upregulating Blimp-1 (4, 6, 24, 30). In fact, we observed that increased IL-2 signaling significantly raised the levels of Blimp1 (Fig. 5H), whereas interfering with IL-2 signaling reduced Blimp1 levels (Fig. 6E). Constitutive STAT5 signaling can selectively block Tfh cell differentiation and GC formation, and this has been shown to be largely mediated by IL-2 signaling (4). However, the relationship between phosphorylated STAT5 and STAT3 levels on these cells still needs to be investigated. Nevertheless, IL-2 could be playing an important role by antagonizing Tfh cell differentiation, and signals through the IL-2R (CD25) could be contributing to shifting...
the balance in favor of Th1–effector differentiation (4, 5, 31, 32). Recent evidence also suggests that defects in the IL-2 pathway leading to suboptimal IL-2 signaling could favor increased Tfh cell differentiation in the setting of type 1 diabetes (33). Increased autocrine IL-2 levels could therefore be affecting the differentiation of Tfh cells and could be partly responsible for the altered functional output in memory Tfh cells from CA subjects. In fact, this Tfh cell defect could contribute to the impaired vaccine responses reported in HIV-infected individuals. For example, it has been shown that the immune response to both hepatitis A and B vaccines seems to be reduced in HIV-infected individuals (34). In addition, the levels of Abs to tetanus toxoid and measles are significantly lower in HIV-infected individuals (35), and (36). Furthermore, it has been shown that some HIV-infected subjects have poor responses to influenza vaccination (37).

However, it is important to note that this increase in IL-2 signaling was not observed across all the CA samples studied and additional factors might also be contributing to the skewing of memory Tfh cell differentiation toward a Th1 phenotype. IFN-γ and TNF-α could also be playing a role in altering and maintaining a Th1-like polarization in memory Tfh cells from CA individuals as well as directly affecting B cell responses. We observed increased levels of both of these cytokines in cocultures from CA subjects as well as blunted B cell help in cocultures from ECs following addition of IFN-γ. The production of these cytokines is likely the result of memory Tfh cell polarization toward a Th1-like phenotype. IFN-γ signaling leads to increased levels of Tbet expression via induction of STAT1 (38). This activity could help tilt the balance in favor of Tbet expression and away from Bcl6. If the levels of Bcl6 go down, this would allow memory Tfh cells to become more polarized toward other effector phenotypes by allowing other transcription factors such as Tbet to outcompete Bcl6 signaling. Indeed, we observed that memory Tfh cells from CA subjects had increased Tbet expression as well as IFN-γ and TNF-α production, molecular targets of Tbet.

Our multiplex Fluidigm BioMark analysis also revealed decreased levels of the canonical Tfh cell cytokine IL-21 in cTfh cells from cocultures of CA individuals when compared with ECs. Indeed, blocking IL-21 signaling in cocultures from HC subjects, using an IL-21R Fc chimera, significantly blunted Ab production.
and the absolute number of live B cells (Supplemental Fig. 3E). Furthermore, supplementation of cocultures from CA subjects with IL-21 significantly increased total IgG Ab output (Supplemental Fig. 3E). The reduced expression of IL-21 in cTfh cells from CA subjects could be partly the result of increased IL-2 signaling on these cells, which could interfere with the STAT3-Bcl6 axis via STAT5 induction, but the overall functional defect in these cells is likely maintained by a combination of signals and altered transcription factor balance. In this sense, increased IL-2 signaling might be playing an important role in helping to interfere with Bcl6 function, which, together with IFN-γ and TNF-α, could further help to drive the altered polarization of memory Tfh cells into a Th1-like phenotype in CA subjects.

How these memory Tfh cells acquire a Th1-like phenotype upon activation is unknown, but it is reasonable to speculate that memory Tfh cells in HIV-infected individuals under ART may continue to experience heightened levels of proinflammatory signals in LNs that, despite ART, can maintain residual continuous local inflammation (16) and viral replication (11). It has been shown that the Tfh cell subset in LNs is a preferential target for HIV infection (39). Because the phenotype of cTfh cells encompasses resting memory T cells, which harbor viral DNA, these cells could also contain viral DNA at extremely low frequencies (one in a million cells) (40, 41). Thus, at this very low frequency, it is possible that the polyclonal function of these cells could not be compromised by viral infection. To confirm this, we performed coculture assays in the presence or absence of ART and found no significant difference in cell viability and Ab production, suggesting that virus infection and replication, at low levels, do not contribute to Tfh dysfunction (data not shown).

Furthermore, it is possible that increased Blimpl expression and IL-2R signaling could be driving these cells toward a regulatory T cell phenotype (42). However, we did not observe a significant frequency of FOXP3+ cTfh cells in cocultures from CA subjects (data not shown), and the increased expression levels of IFN-γ and TNF-α suggest that these cells are not acquiring a T follicular regulatory T cell/regulatory T cell–like phenotype.

It is also important to point out that in this study we investigated only the phenotype and function of cTfh cells in peripheral blood and not LN-resident Tfh cells, owing to the difficulty of obtaining lymphoid tissues from HIV-infected individuals and HCs. It is still unclear to what extent memory Tfh cells represent their LN counterparts. Memory Tfh cells could generate from pre-Tfh cells before these cells enter the GC or could be derived from GC Tfh cells. However, the exact identity of these cells is still under investigation. For this reason we cannot conclude that what we observe in cTfh cells is exactly what is occurring to Tfh cells in LNs, but by investigating these cells we can infer what general microenvironmental conditions were present in lymphoid tissues when these cells were differentiated. This understanding provides a glimpse into the conditions experienced in LNs by both of these cell types, which could affect their overall differentiation and polarization. Our findings therefore provide an insight into the mechanisms responsible for the induction and maintenance of Tfh cell differentiation that could be important in vaccine development. Enhancing memory Tfh cell function could also help overcome various humoral immune defects; enhance Ab effector functions; and improve the affinity maturation of HIV-specific Abs in HIV-infected individuals, which could potentially lead to an improved control of viral replication.

Acknowledgments

We thank Kim Kusser and Yu Shi from the Flow Cytometry Core at Vaccine and Gene Therapy Institute Florida for expertise and help in sorting all the samples; William Williams and Nicole L. Yates for expertise with the Ab subclass assays; Drs. H. Liao and B. Haynes for HIV-1 envelope proteins and mAbs; and all the patients who provided samples, without the partial support of which this study would not have been possible.

Disclosures

The authors have no financial conflicts of interest.

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