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Circulating CXCR5⁺PD-1⁺ Response Predicts Influenza Vaccine Antibody Responses in Young Adults but not Elderly Adults


Although influenza vaccination is recommended for all adults annually, the incidence of vaccine failure, defined as weak or absent increase in neutralizing Ab titers, is increased in the elderly compared with young adults. The T follicular helper cell (Tfh) subset of CD4 T cells provides B cell help in germinai centers and is necessary for class-switched Ab responses. Previous studies suggested a role for circulating Tfh cells (cTfh) following influenza vaccination in adults, but cTfh have not been studied in elderly adults in whom weak vaccine responses are often observed. In this study, we studied cTfh expressing CXCR5 and programmed death-1 (PD-1). cTfh from elderly adults were present at reduced frequency, had decreased in vitro B cell help ability, and had greater expression of ICOS compared with young adults. At 7 d after inactivated influenza vaccination, cTfh correlated with influenza vaccine–specific IgM and IgG responses in young adults but not in elderly adults. In sum, we have identified aging-related changes in cTfh that correlated with reduced influenza vaccine responses. Future rational vaccine design efforts should incorporate Tfh measurement as an immune correlate of protection, particularly in the setting of aging. The Journal of Immunology, 2014, 193: 3528–3537.

Annual vaccination against influenza A is recommended for all individuals >6 mo of age, but vaccine efficacy for the inactivated vaccine in young, healthy adults is only 60%, depending on the year (1). For adults over age 65, vaccine efficacy may be 0–60% (2–4). Moreover, 90% of influenza-related deaths occur among adults over age 65 (5). Thus, there is a strong rationale for improved influenza vaccines for use in the elderly. The etiology of poorer vaccine effectiveness in the elderly as compared with younger adults is multifactorial (6–8). For the influenza vaccine, the primary correlate of protection is the development of strain-specific neutralizing Abs (9–11). A variety of defects in Ab production have been identified in the elderly, including reduced Ab diversity (12), reduced numbers of vaccine-induced plasmablasts (13), and abnormal activity of APCs (14). These factors together result in weak vaccine responses in a population already at high risk for morbidity and mortality.

To produce high-affinity, class-switched Abs, B cells in germinal centers require help from the lymphoid T follicular helper cell (Tfh) subset of CD4 T cells (15). This subset was first identified in human tonsillar tissue and peripheral blood based on the chemokine receptor CXCR5 (16). Expression of CXCR5 increases after stimulation of naive cells, which likely permits trafficking of these cells to the B cell zones of lymphoid tissue (17, 18). Tfh interact with other cells via a number of different molecules including programmed death-1 (PD-1), signaling lymphocytic activation molecule–associated protein (SAP), cytotoxic receptors, costimulatory molecules, and others (19–25). Absence of Tfh leads to immune dysregulation and a failure to develop memory B cells (19, 26–28). In the setting of HIV infection, increased Tfh activity leads to dysregulated Ab production (29) and contributes to poor vaccine-induced IgG responses (30). Because nearly all clinically available vaccines rely on Ab production for maximal effect,

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R.S.H., D.V.D., and K.D.M. designed and performed experiments on cohort 1. R.S.H. and E.J.W. conceived the overall study design and wrote the paper; M.A.R., H.A., O.Z.B., M.R.B., and D.H.C. conceived and performed experiments related to cohort 2. R.K.K., S.K., and H.E. performed antibody assays. K.E.S. and D.H.C. performed patient accrual. All authors analyzed and interpreted data, discussed the results, and commented on the manuscript.

Address correspondence and reprint requests to Dr. E. John Wherry, University of Pennsylvania, Perelman School of Medicine, 421 Curie Boulevard, BRB II/III Room 354, Philadelphia, PA 19104. E-mail address: wherry@mail.med.upenn.edu

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Abbreviations used in this article: cTfh, circulating Tfh cell; HAI, hemagglutinin inhibition assay; MN, microneutralization; PD-1, programmed death-1; QRT-PCR, real-time quantitative RT-PCR; SAP, signaling lymphocytic activation molecule–associated protein; SEB, staphylococcal enterotoxin B; SEF, staphylococcal enterotoxin F; Tfh, T follicular helper cell.

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generation of balanced Th responses will be critical to improved vaccines.

Recent efforts have focused on evaluating circulating Tfh-like cells (cTfh). A subset of circulating memory CD4 T cells was identified that expressed CXCR5, had the ability to provide B cell help in vitro, and was expanded in the setting of chronic inflammatory conditions (31–35). However, circulating CD4+CXCR5+ cells lacked increased Bcl-6 expression, a marker of germinal center Tfh, and were different from lymphoid Tfh in microarray analyses (31, 33). A subset of these, identified as CD4+CXCR5+ CXCR3+CCR6+, expressed PD-1 but not Bcl-6, responsive to influenza vaccination, and correlated with neutralizing Ab responses in adults but not children (36). Recently, Locci et al. (37) identified CXCR5+CXCR3 PD-1+ cells as being highly similar to lymphoid Tfh; these cells correlated with neutralizing Ab production against HIV. In contrast, Spensieri et al. (38) identified an expanded subset of CD4+ cells 21 d postinfluenza vaccination that expressed IL-21 and PD-1 after in vitro stimulation, lacked CXCR5 expression, and correlated with neutralizing Ab titters. Thus, the precise definition of cTfh continues to evolve in human studies. Understanding the relationship of these cells to different vaccine outcomes between young and elderly individuals holds promise for improving vaccines against pathogens such as influenza.

Given the deficiencies in influenza vaccine responses observed in the setting of aging, we hypothesized that abnormal cTfh responses in the elderly may underlie poor vaccine responses when compared with young adults. In this study, we found that the elderly have 35% fewer cTfh, and these cells expressed greater levels of ICOS compared with cells from young adults. Moreover, we observed that cTfh from the elderly had a per-cell decrease in functional ability to help B cells compared with cTfh from young adults. Influenza vaccination induces a clear increase in ICOS expression in cTfh from young adults but only a weak increase in the elderly, suggesting defects in vaccine-induced Tfh activation. This change in ICOS is predictive of vaccine-induced IgM and IgG responses in young adults but not elderly adults. In summary, we identify aging-related changes in cTfh that are associated with reduced influenza vaccine-induced Ab responses. Future studies of cTfh as a T cell–based immunological correlate of protection are warranted.

Materials and Methods

Human subjects for cohort 1

In the fall of 2012, study subjects were recruited and consented at the Clinical Research Unit at Duke University Medical Center (Durham, NC), in accordance with the Institutional Review Boards of both Duke University and the University of Pennsylvania (Philadelphia, PA). Subjects were classified as young (30–40 y of age) or elderly (>65 y of age). Subjects were excluded if they had contraindications to influenza vaccine, active substance abuse, HIV/AIDS, clinically active malignancy, immunomodulatory medication need (i.e., chemotherapy or corticosteroids), active intercurrent illness (i.e., active respiratory tract infections), or were on cortisone medication need (i.e., chemotherapy or corticosteroids), active intercurrent illness. Demographic data were collected at baseline on all subjects.

Peripheral venous blood was drawn into heparinized tubes for PBMC isolation.

Flow cytometry

PBMC and plasma were isolated using Ficoll-Paque PLUS (GE Healthcare), rested overnight at 37 °C in RPMI 1640 containing 10% FCS, and stained for surface and intracellular markers. The following Ab conjugates were used in cohort 1: Violet and Aqua LIVE/DEAD (Invitrogen); CCR7-Pacific Blue (G043H7; BioLegend); CD14-V500, CD16-V500, CD19-V500, and CD19-PE-Cy7 (BD Biosciences); CD126-PE-Cy5 (BioLegend); CD38-PE-Cy5 (BioLegend); CD62L-PE–Texas Red (BD Biosciences); ICOS-PE-Cy7 (C398.4a; BioLegend); CXCR5–Alexa Fluor 647 (RF8B2; BD Biosciences); CD8-APC-eFluor780 (eBioscience); and CD3-Qdot 585 (custom conjugated). Permeabilization was performed using the Foxp3 Fixation/Permeabilization Concentrate and Diluent kit (eBioscience) and intracellular stains were done with Bc16-PerCP–eFluor 710 (BCL-UP; eBioscience); and Ki67-FITC (BD Biosciences). For cohort 2, additional conjugates included used PD-1–BV421 (BioLegend); CCR7-BV711 (Bio-Legend); CD3-BV570 (BioLegend); CD8-Qdot 605 (Invitrogen); CD27-Qdot 655 (Invitrogen); CXCR5–Alexa Fluor 488 (BD Biosciences); CD8-APC–Texas Red (Beckman Coulter); CD4-PE-Cy5 (Invitrogen); and CD16-PE-Cy5 (BioLegend). Permeabilization was performed using the Cytofix/Cytoperm kit (BD Biosciences). ICOS clone comparison was performed against ICOS-APC–eFluor 780 (ISA-3; eBioscience). Cells were resuspended in 1% paraformaldehyde until acquisition on an LSR II cytometer (BD Biosciences) and analyzed using FlowJo (Tree Star).

Fluorescence-minus-one controls were performed in pilot studies.

IL-21 by flow cytometry

PBMC were stimulated for six hours in the presence of 1 µg/ml staphylococcal enterotoxin B (SEB) or left unstimulated as a control. Brefeldin A was added for the final 5 h of stimulation. Staining was performed at 37 °C in the stimulation medium followed by fixation with 1% paraformaldehyde for 10 min at room temperature. Cells were then permeabilized with the Foxp3 Fixation/Permeabilization Concentrate and Diluent kit and intra-cellular staining with IL-21 (3A3-52; eBioscience) was performed for 1 h at room temperature.

Real-time quantitative RT-PCR

Sorted PBMC were lysed and RNA isolated using the RNeasy Mini Kit (Qiagen) followed by reverse transcription (Applied Biosystems). Real-time quantitative RT-PCR (QRT-PCR) was performed using the TaqMan Universal PCR Master Mix (Roche) and predesigned primers and hydrolysis probes from IDT (Corvalle, IA) for BCf6, BT1A, CD200, SH2D1A, and IL21. Data were normalized against the reference gene β2M and expressed as fold change compared with naive.

B cell coculture assays

Sorted CD4 T cell subsets were plated with either autologous (sorted as CD3+CD19+IgM–CD27+CD38+) or allogenic naive B cells (Miltenyi Naive B cell Isolation Kit; Miltenyi Biotec) in a 1:1 ratio and cocultured for 7 d in the presence or absence of 0.1 µg/ml SEB or staphylococcal enterotoxin F (SEF; Toxin Technology). Cells were analyzed by flow cytometry as above. Supernatants were analyzed by ELISA. Briefly, Nunc Maxisorp plates (Nunc) were coated with goat anti-human IgG+IgM (Jackson ImmunoResearch Laboratories) or goat anti-human IgM or IgG (Abcam and Bethyl Laboratories, respectively). Bound Abs were detected by biotinylated anti-human IgM or IgG (Jackson ImmunoResearch Laboratories) or HRP-conjugated mouse anti-human IgM or IgG (Southern Biotechnology Associates and Bethyl Laboratories, respectively).

Ab responses

The two influenza A vaccine strains of the 2012/2013 seasonal influenza vaccine, A/California/7/2009 (H1N1) pdm09-like virus and A/Victoria/361/2011 (H3N2)-like virus, were obtained from the Centers for Disease Control and Prevention (Atlanta, GA). Assays to detect hemagglutinin inhibition assay (HAI) titers and microneutralization (MN) titers were performed (39). Infectious virus was used for neutralizing Ab assays or inactivated by β-propiolactone for H1N1-California- and H3N2-Victoria-specific binding Abs. Allophycocyanin-labeled Maxisorp plates (Nunc) were coated with 10 µg/ml influenza A/H1N1/California and A/H3N2/Victoria virus along with isotype standards for IgG1, IgG2, and IgM (Athen’s Research and Technology) in bicarbonate buffer overnight at 4 °C. Plates were blocked with 3% BSA in bicarbonate buffer overnight at 4 °C.
CBS and incubated with heat-inactivated sera of young and elderly subjects. Abs were detected using alkaline phosphatase-conjugated mouse anti-human IgA, IgG, and IgM (Southern Biotechnology Associates).

Statistics

Statistical analyses were performed with Excel (Microsoft) and Prism (GraphPad). Data was compared using Student t test, paired t test, or one-way ANOVA with Tukey post hoc analysis.

Results

cTfh-like cells are similar to lymphoid Tfh cells

Lymphoid Tfh cells express many markers including CXCR5, ICOS, PD-1, SAP, Bcl-6, and others (15, 25). Given the importance of ICOS expression for Tfh function (27, 28, 33, 40), we defined cTfh cells as nonnaive CD4+CXCR5+PD-1+ based on previous work (41, 42) and observed increased expression of ICOS compared with other subsets (Fig. 1A, 1B, Supplemental Fig. 1A–F). To further probe the relationship between cTfh cells and previously described lymphoid Tfh (25), we undertook extended phenotyping for additional Tfh markers. cTfh cells demonstrated increased expression of CD126 (IL-6Rα) (Fig. 1C), decreased expression of CCR7 (Fig. 1D), and increased expression of CXCR4 (Fig. 1E) compared with naive cells, all of which were consistent with prior studies of lymphoid Tfh (25). Furthermore, cTfh cells demonstrated greater expression of CD126 and CXCR4 than nonnaive CXCR5+ cells. These data suggested high phenotypic similarity between lymphoid Tfh and cTfh cells.

To further interrogate this relationship, transcriptional analysis was performed on sorted CD4 + T cell subsets. Analysis by qRT-PCR demonstrated increased transcripts for IL21, SH2D1A (SAP), and CD200, but not BTLA, compared with naive CD4+ cells (Fig. 1F). We further confirmed that CD200 protein expression was higher in cTfh than in nonnaive CXCR5+ cells (p < 0.001) at baseline and that BTLA protein expression was not different between CD4 subsets (data not shown). Interestingly, Bcl6 protein expression was not different between cTfh cells and CXCR5+ nonnaive cells by flow cytometry (data not shown) but cTfh had 2-fold higher BCL6 mRNA expression than naive CD4+ cells.

cTfh have an intrinsic ability to provide B cell help in vitro, despite lower Bcl6 protein expression than their lymphoid counterparts (31, 32, 36, 37). Coculture of sorted autologous naive B cells with sorted cTfh, nonnaive CXCR5−, or naive cells for 7 d was performed in the presence of supernatant. Coculture with cTfh resulted in greater production of IgM and IgG in the supernatant (Fig. 1G) and greater B cell differentiation to plasma cells (Fig. 1H, Supplemental Fig. 1G) compared with coculture with naive or nonnaive CXCR5− CD4+ cells. Together, these data suggest a strong resemblance between circulating cTfh cells and lymphoid Tfh cells based on phenotype, transcriptional analysis, and functional ability.

cTfh from elderly subjects demonstrate reduced circulating frequency and increased expression of ICOS

We hypothesized that differences in cTfh between young and elderly adults might contribute to weaker vaccine responses often seen in the elderly. To test this, we assessed cTfh frequency at baseline in a cohort (cohort 1) of young (ages 30–40 y; n = 28) and elderly (ages 66–88 y; n = 35) adults (Supplemental Table I). An average of 2.18% of nonnaive CD4+ T cells were CXCR5+PD-1+ in the young adult cohort, whereas only 1.12% were CXCR5+PD-1+ in the nonnaive CD4+ T cells of the elderly (p = 0.033) (Fig. 2A, 2B).

We then examined cTfh in a second, independently recruited cohort (cohort 2) of young (n = 21; ages 22–54 y) and elderly (n = 26; ages 63–90 y) adults (Supplemental Table I). Once again, there were fewer circulating cTfh in the elderly compared with young adults (p = 0.016; Supplemental Fig. 2A). Based on the combined cohorts, elderly adults had a 35% reduction in cTfh frequency compared with young Adults (Fig. 2C).

To determine whether there might be other differences between young and elderly adults, we assessed expression of ICOS in cTfh. Interestingly, cTfh from elderly adults had greater baseline expression of ICOS in both cohorts 1 and 2 (p = 0.042 and p = 0.0003, respectively). Thus, we found fewer circulating cTfh in the elderly compared with young adults, but cTfh from the elderly expressed more ICOS.

cTfh strongly coexpress Ki67 and CD38 but have reduced functional ability in the elderly

To address whether reduced proliferation could explain the reduced frequency of cTfh in elderly adults, we measured coexpression of Ki67 and CD38, which have been previously used to identify proliferating cells after vaccination (43–45). Nonnaive CXCR5+ cells had weak coexpression of these markers, whereas cTfh showed much greater coexpression of Ki67 and CD38 (Fig. 3A, 3B, Supplemental Fig. 2C). Furthermore, ICOS−cTfh had even greater expression of Ki67 and CD38. There was no apparent difference in expression of Ki67 and CD38 in cTfh or ICOS−cTfh cells between young and elderly adults in the steady state, however (Fig. 3B).

To determine whether functional ability of cTfh differed in young and elderly adults, sorted allogeneic naive B cells (>90% IgD+IgM+) from a young subject were cocultured with sorted cTfh cells from young or elderly subjects in the presence of SEB for 7 d. Measurement of IgM and IgG production in the supernatant by ELISA revealed similar IgM levels in cultures containing cTfh from either young or elderly subjects; however, IgG levels were decreased in cultures containing cTfh from the elderly, compared with cTfh from young subjects (Fig. 3C). In the absence of SEB, IgG production in cultures with cTfh from the young compared with the elderly was substantially lower, but not different between the groups (112 and 251 ng/ml, respectively; p = 0.31, data not shown).

Thus, cTfh from the elderly demonstrated reduced B cell help ability on a per-cell basis, compared with cTfh from young adults.

Because IL-21 production is a key property of Tfh, we asked whether cTfh from the young and elderly were equally capable of making IL-21 protein. After 6 h of SEB stimulation, cTfh produced more IL-21 than memory CXCR5− CD4+ cells (Fig. 3D, 3E). However, we did not observe any difference between cTfh from young or elderly subjects in IL-21 expression (p = 0.54) or in mean fluorescence intensity (Supplemental Fig. 2B) in a representative subgroup of subjects. This result suggests that despite a numerical reduction, cTfh from the elderly retain the ability to produce this important B cell help cytokine.

To determine whether alteration of CD4 T cell responses to SEB with age (e.g., perhaps due to age-related TCR repertoire changes) could explain the reduced B cell help provided by cTfh from elderly subjects, PBMC were stimulated with SEB, SEF, or PMA/ionomycin in vitro for 6 h and assayed by flow cytometry. No difference in the frequency of CD4+IFN-γ+ cells was observed between young and elderly subjects for any stimulation condition (data not shown), suggesting an equal proportion of CD4+ T cells in young and elderly are capable of responding to superantigen stimulation by producing IFN-γ.

Influenza vaccine induces increased ICOS expression in cTfh in the young but not elderly

We were next interested in whether the differences in cTfh frequency and functional ability described above would result in altered cTfh responses to influenza vaccination. Subjects from
cohort 1 (28 young and 35 elderly subjects) were given seasonal trivalent inactivated influenza vaccine and followed by serial phlebotomy on days 0, 7, and 14. Samples were analyzed by flow cytometry for cTfh responses (Fig. 4A, 4B).

Similar to a prior study (36), there was no change in circulating cTfh frequency after vaccination in young or elderly adults (Fig. 4C, 4F). However, ICOS expression on cTfh increased in the young ($p = 0.0031$) (Fig. 4A, 4D), but only showed a trend toward
increase in the elderly ($p = 0.094$, Fig. 4B, 4G). There was a stronger trend toward increased ICOS$^+$CD38$^{hi}$ in the young than in the elderly (Fig. 4A, 4B, 4E, 4H). We then assessed whether there was a difference between young and elderly in proliferation following vaccination based on Ki67$^+$CD38$^{hi}$ coexpression. We observed increased coexpression of both markers after vaccination when analyzed across the whole cohort (Fig. 4L–L). Surprisingly, observed increased coexpression of both markers after vaccination from young subjects (Fig. 4K). Analysis of ICOS$^+$ cTfh (Fig. 4L) revealed a small but statistically significant increase in coexpression of these markers in cTfh from the young ($p = 0.04$) and a larger increase in cTfh from the elderly ($p = 0.011$). These data suggest that cTfh from the elderly, and in particular the ICOS$^+$ subset, had recently entered cell cycle to a greater extent than in young subjects, but this proliferation did not result in increased cTfh circulating frequency overall or in ICOS expression. In sum, cTfh from young but not elderly adults showed an increased expression of ICOS following influenza vaccination.

Change in ICOS expression in cTfh correlated with Ab production in the young but not elderly

We hypothesized that the cTfh response would predict the vaccine-induced Ab response in young but not elderly adults. Prior studies have correlated neutralizing Abs with the Tfh response in adults (36, 46). Others have evaluated total IgM and IgG responses to influenza vaccine and observed variability depending on vaccine year (47–49). Nevertheless, evaluation of the total humoral response is important for understanding the overall impact of the cTfh response, as neutralizing Abs likely represent a very narrow slice of the overall Ab response to vaccination.

To assess whether Tfh correlated with Ab responses, we evaluated titers of the influenza vaccine-induced IgM and IgG response in all subjects. The fold change for influenza A strains (H1N1/Cal and H3N2/Vic) were averaged. At baseline, young and elderly adults had similar circulating levels of influenza-specific IgM and IgG Abs ($p = 0.18$ and $p = 0.58$, respectively; Fig. 5A–D). Following influenza vaccination, total influenza-specific IgM and IgG responses were 3.5- and 1.2-fold greater in young adults at day 7 compared with day 0, respectively (Fig. 5A, 5B), which was similar in range to that previously reported (47, 49). Both isotypes were still slightly higher (3.6- and 1.2-fold, respectively) at day 14 compared with day 0. Elderly adults had a 2.38-fold increase of IgM at day 7 and 2.44-fold increase at day 14 over day 0 (Fig. 5C). However, IgG in the elderly was only 1.05-fold greater at day 7 and day 14 compared with day 0 (Fig. 5D). Thus, the influenza vaccine led to a robust IgM response in the young adults and a slightly weaker response in the elderly, whereas the vaccine led to a weak IgG response in the young and a nearly absent IgG response in the elderly.

We then assessed whether the peak cTfh response would correlate with the influenza-specific Ab response. Prior studies have identified a correlation between cTfh expression of ICOS and neutralizing Ab titers at day 28 (36). MN at day 14 showed a correlation between the ICOS response in cTfh in young ($r = 0.51; p = 0.009$) but not elderly ($r = 0.13; p = 0.47$) subjects (Supplemental Fig. 3A), whereas HAI at day 14 did not correlate with the ICOS response in cTfh in young or elderly subjects (Supplemental Fig. 3B).

However, we reasoned that HAI and MN only identify a small proportion of the total Ab pool generated by influenza vaccination. We assessed whether the cTfh response would correlate with the total influenza-specific IgM and IgG response. There was no correlation between the change in circulating frequency of cTfh and the total IgM or IgG response of either cohort (data not shown) (36). However, there was a significant correlation between the change in the ICOS$^+$-cTfh population and the total IgM and IgG responses in the young adults (Fig. 5E, 5F). In contrast, there was only a weak, nonsignificant trend observed with IgM responses in the elderly and no correlation at all with IgG responses (Fig. 5G, 5H). To exclude a later Ab response, we compared day 7 cTfh responses to day 14 Ab responses but found weaker associations in young subjects and no association in elderly subjects (Supplemental Fig. 3C). Finally, we found no
correlation between changes in Ki67 and CD38 coexpression and IgM or IgG Ab production in subsets of young and elderly subjects (data not shown). Together, these data suggest that deficient immune responses in the elderly, as manifested by reduced cTfh activation, could underlie decreased Ab responses.

Discussion

Understanding Tfh cells is key to developing strategies to optimize vaccine responses. We have identified clear differences in cTfh cells between young and elderly adults. There was no age-related difference in expression of CD126, CXCR4, or CCR7, nor was there any age-related difference in transcripts for IL21, SH2D1A, CD200, or BTLA (data not shown). However, at baseline, elderly adults had a 35% reduction in the circulating frequency of cTfh compared with young adults. The etiology for this decrease was not clear but did not appear to be due to reduced proliferation, based on the analysis of CD38 and Ki67 expression. Although reduced in frequency, cTfh in elderly adults had higher expression of ICOS and coexpression of Ki67 and CD38 after influenza vaccination, compared with young adults. However, increased activation did not lead to strong vaccine-induced IgG Ab responses in the elderly. Moreover, direct analyses of cTfh helper function revealed a per-cell decrease in B cell help ability. That, coupled with the reduced circulating frequency in nonnaive CD4 in the elderly, could have an impact on vaccine responsiveness. In addition to reduced vaccine-induced plasmablasts (13) and abnormal APC activity (14), poor cTfh activity should be considered in the multifactorial picture of vaccine nonresponses in the elderly.

ICOS is important for Tfh–B cell interactions (27, 40); increased ICOS expression coupled with reduced function suggest there may be broader, more fundamental age-related changes in cTfh. Of note, inflammation has been strongly correlated with, and may be a direct cause of, immune senescence (7). The increased ICOS expression at baseline in the elderly may also reflect chronic, non-specific inflammation. Furthermore, IL-6, which is highly relevant to Tfh (23, 24), has been implicated as a direct inducer of senescence in other settings (50). The age-related changes in cTfh are unlikely to be sex or ethnicity related, given the demographically different cohorts in the current study.

Despite increased expression of activation markers at baseline, cTfh from the elderly were less efficient at helping B cells in vitro on a per-cell basis. Aging-related B cell dysfunction has been
described (51). To control for this issue, we used a single source of allogeneic naive B cells from a young subject. Therefore, these B cell help assay results focus attention on changes in cTfh with age. Although the reduced ability to produce IL-21 was implicated in reduced B cell help ability of cTfh in the setting of HIV (52), we did not find the same to be true in the elderly compared with young adults. However, the kinetics and/or efficiency with which IL-21 protein or other help-related proteins such as CD40L are produced locally and made available to B cells, or the ability to form cTfh–B cell conjugates during prolonged stimulation, should be important areas for future investigation. In sum, we have identified age-related changes in circulating frequency, ICOS expression, and B cell help ability in cTfh that may have important implications for Ab generation.

**FIGURE 4.** After influenza vaccination, cTfh show increased ICOS expression in young but not elderly adults. (A and B) Influenza vaccine was administered to young (n = 28) and elderly (n = 35) adults followed by serial phlebotomy. cTfh were measured by flow cytometry. Plots indicate CD38 versus ICOS expression of cTfh for one young (A) and one elderly (B) subject at days 0 and 7 after vaccination. Red numbers indicate frequency of ICOS^CD38^hi^ cTfh among nonnaive CD4^+^ T cells for each time point. Frequency of cTfh after influenza vaccination in young adults (n = 28; C) and elderly adults (n = 35; F) are shown. ICOS expression in cTfh from young adults (D) and elderly adults (G) are shown. Coexpression of ICOS and CD38 in cTfh cells from young adults (E) and elderly adults (H) are shown. Coexpression of Ki67 and CD38 in cTfh is shown for one representative young (I) and elderly (J) subject at days 0 and 7. Summary plots of coexpression of Ki67 and CD38 in cTfh (K) and in ICOS^+^ cTfh (L) cells are shown. The p values indicate results of paired t test analyses comparing days 0 and 7.
In our study, we observed higher expression of ICOS at baseline using the C398.4A clone than others have previously reported using the ISA-3 clone (37, 53). The C398.4A clone binds a different epitope than ISA-3 (54, 55) and is known to exhibit stronger baseline staining (Supplemental Fig. 1E, 1F). Young and elderly samples were treated the same in all experiments. Furthermore, we did not observe differential nonspecific activation effects from the overnight rest prior to staining (Supplemental Fig. 1D). Nonetheless, the ICOS clone and appropriate controls should be carefully considered.

In the current study, cTfh have phenotypic and functional properties that strongly resemble those of lymphoid Tfh. At baseline, cTfh demonstrated expression of CD126, ICOS, PD-1, and CXCR4, had increased mRNA transcripts for IL21, SH2D1A, and CD200, and provided B cell help activity in vitro, which are all consistent with prior studies (25, 31, 32, 36). There were, however, notable discrepancies between lymphoid Tfh and the cTfh subset. In our study, cTfh had increased mRNA transcripts for BCL6 over naive cells but not increased protein expression. Discordance between BCL6 mRNA and Bcl-6 protein expression has been previously reported (56, 57). Moreover, prior studies have not detected elevated Bcl-6 protein expression in PBMCs expressing CXCR5+ compared to CD4 subsets (31, 33, 34, 36). In addition, BTLA has been reported as a lymphoid Tfh marker (25), but we did not detect elevated BTLA mRNA in cTfh cells. In a recent study, Locci et al. (37) identified CXCR5+PD-1+ CXCR3+ cells as being highly similar to germinal center Tfh based on microarray analyses; this particular subset was better than other CXCR5+ subsets in providing in vitro B cell help. Our studies are consistent with recent data (36, 37). We further extend the emerging definition of cTfh and provide strong evidence that cTfh defined as CD4+CXCR5+PD-1+ possess B cell help capacity.

However, the precise relationship of these cTfh to lymphoid Tfh during germinal center responses has not been fully elucidated in humans. Nonetheless, our results demonstrate appropriate cTfh responses can be a useful predictor of vaccine-induced Ab responses.

New questions arise from our findings. First, a prior report demonstrated a correlation between changes in ICOS expression at day 7 in CD4+CXCR5+CXCR3+CCR6+ cells with both HAI titer and MN titers measured at day 28 (36). Vaccine responses are typically assessed using day 28 titers. Less is known about HAI titers before day 28, although neutralizing Ab production was higher at 14 d after influenza vaccination compared with 7 d (58). We assessed HAI and MN titers but did not find consistent correlations with changes in ICOS expression in cTfh or circulating frequency early after vaccination, although MN titers did correlate at day 14 after vaccination. Our measurements of influenza-specific IgM and IgG are similar to a previous report at day 7 after vaccination, but these values vary depending on the vaccine strain and by year (47, 49).

Second, the importance of Ag specificity is unknown in the cTfh response. cTfh in other studies produced few cytokines, making this approach to identify Ag-specific cells problematic (36, 37). Future studies to address Ag specificity of cTfh may require tetramer reagents or phospho-flow studies. Given the observation of preferential boosting of neutralizing Ab titers over many years (59), seasonal influenza vaccination may also preferentially boost only certain cTfh responses. However, CD4 responses may be directed against conserved internal proteins (60), whereas strong HAI responses could require hemagglutinin-specific cTfh.

Third, our data identified increased Ki67 expression in cTfh from the elderly compared with the young after vaccination. The
reasons for the increased proliferation of cTfh in the elderly are unclear. Lymphoid Tfh have a limited proliferation capacity (33, 41, 61, 62), which may be due to expression of inhibitory receptors such as PD-1 (63). Paradoxically, excess proliferation in cTfh in the elderly may be evidence of dysfunction, reflecting compensation for reduced frequency, reduced diversity in the TCR repertoire with aging (64), or reduced ability to provide B cell help on a per-cell basis, as compared with young adults. Although differences in cTfh between young and elderly adults were observed here, it will be important to extend these observations to lymphoid tissues where help to B cells is actually delivered and neutralizing Abs are generated.

Successful influenza vaccines are predicated on increased production of high-avidity, neutralizing Abs. The generation of such Abs requires the efficient cooperation of several arms of the immune response including Ag-specific B cells and Tfh. In addition to other age-related immunological changes, the ability of CD4 T cells to provide help to B cells and induce robust Abs responses may decline in older humans. These data, therefore, suggest that future vaccine studies should evaluate cTfh responses as an additional correlate of protection. Our findings highlight the importance of developing vaccine strategies that boost Tfh responses to improve vaccine responsiveness in the elderly.

Disclosures
The authors have no financial conflicts of interest.

References


