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IL-4–Secreting Secondary T Follicular Helper (Tfh) Cells Arise from Memory T Cells, Not Persisting Tfh Cells, through a B Cell–Dependent Mechanism

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Humoral immunity requires cross-talk between T follicular helper (Tfh) cells and B cells. Nevertheless, a detailed understanding of this intercellular interaction during secondary immune responses is lacking. We examined this by focusing on the response to a soluble, unadjuvanted, pathogen-derived Ag (soluble extract of *Schistosoma mansoni* egg [SEA]) that induces type 2 immunity. We found that activated Tfh cells persisted for long periods within germinal centers following primary immunization. However, the magnitude of the secondary response did not appear to depend on pre-existing Tfh cells. Instead, Tfh cell populations expanded through a process that was dependent on memory T cells recruited into the reactive LN, as well as the participation of B cells. We found that, during the secondary response, IL-4 was critical for the expansion of a population of plasmablasts that correlated with increased SEA-specific IgG1 titers. Additionally, following immunization with SEA (but not with an Ag that induced type 1 immunity), IL-4 and IL-21 were coproduced by individual Tfh cells, revealing a potential mechanism through which appropriate class-switching can be coupled to plasmablast proliferation to enforce type 2 immunity. Our findings demonstrate a pivotal role for IL-4 in the interplay between T and B cells during a secondary Th2 response and have significant implications for vaccine design. ***The Journal of Immunology*****, 2015, 194: 2999–3010.**

T follicular helper (Tfh) cells are a critical subset of $CD4^+$ T cells that are specialized to provide cognate help to B cells (1). Tfh cells express CXCR5, allowing them to access B cell follicles, where they participate in germinal center (GC) development and secrete cytokines (e.g., IL-21, IL-4, and IFN-γ) that drive both B cell proliferation and Ig class switching to allow the production of IgG1/IgE (IL-4) and IgG2a (IFN-γ) (2–4). Tfh cell and GC B cell numbers are tightly correlated, and the two cell types appear to be able to support each other's prolonged persistence as long as Ag is available (5). Developmental studies revealed that Tfh cells express a distinct repertoire of genes and can develop in vivo where conditions for Th1, Th2, or Th17 cell development are impaired (6, 7). These types of studies led to the conclusion that Tfh cells are a distinct lineage. Other studies, including our own, suggest that in type 2 immunity, Tfh cells emerge from cells that are already committed to the Th2 lineage and, therefore, can be regarded as a specialized subset of these cells (8, 9). However, the relatedness of Tfh cells to Th2 cells in type 2 immunity has been questioned, especially in light of the fact that IL-4, a key marker of Th2 cells, has also been defined as a marker of Tfh cells (10). It has been unclear how this situation could be compatible with the preferential induction of IgG2a during type 1 immune responses. On a related issue, although the role of IL-4 in the primary type 2 response is well documented (11, 12), its role, if any, in a secondary type 2 response, which presumably involves the reactivation of memory B cells that are already class-switched, remains unclear.

As is the case with other helminth parasites, infections with the parasite *Schistosoma mansoni* lead to strong type 2 immunity; much of this response is induced by, and directed toward, Ag secreted by the egg stage of the parasite (13, 14). Type 2 immunity in this infection involves the development of Th2 cells, IL-4–producing Tfh cells, and IgG1-producing B cells, which together play important protective roles during infection (15, 16). Intriguingly, a soluble extract of *S. mansoni* egg (SEA) is able to induce strong Th2 and Tfh responses in the absence of additional adjuvant (8), allowing us to study natural immune responses without the confounding factors of infection.

Recently, there has been considerable interest in the nature of secondary Tfh cell responses. Recent work revealed that, following Ag clearance, Tfh cells possess the capacity to differentiate further into a resting memory $CD4^+$ T cell pool. The properties of these memory cells remain unclear, because some reports showed that, upon rechallenge, they retain their Tfh lineage commitment (17), whereas others showed that, depending on the nature of the secondary response, they possess the ability to differentiate into Th effector cells (18). The situation is complicated by the fact that, in a few reports, Tfh cells were shown to persist following primary immunization, and it was suggested that these cells serve as lymphoid reservoirs of Ag-specific memory Tfh cells (19). However,

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Abbreviations used in this article: ASC, Ab-secreting cell; GC, germinal center; LN, lymph node; SEA, soluble extract of *Schistosoma mansoni* egg; Tfh, T follicular helper; WT, wild-type.

whether these cells truly are memory cells is debatable, because it is now clear that maintenance of the Tfh cell phenotype requires GC B cells and persistent Ag (5), suggesting that if Tfh cells are detected late after immunization, it is because they are continuing to be stimulated by Ag. The possibility that Tfh cells arise from memory T cells following secondary immunization raises the question of whether B cells play a role in this process as they do in the generation of primary Tfh cell responses (1).

In this study, we explored the development of Tfh cells during a secondary response to unadjuvanted SEA, focusing on the role of persistent Tfh cells versus committed memory cells in this process. We further asked whether B cells play a role in secondary Tfh cell responses and explored the function of IL-4 during the secondary type 2 response. Our data suggest that, in the course of a secondary type 2 response, Tfh cells arise largely from memory T cells and not persisting Tfh cells, and that this process is dependent on non-GC B cells. Additionally, we found that IL-4 production is coupled to IL-21 production in Tfh cells in secondary type 2, but not type 1, responses, and that IL-4 plays a critical role in supporting the rapid outgrowth of plasmablasts that characterize this response.

Materials and Methods

Mice and parasites

C57BL/6, BALB/c 4get/KN2, and C57BL/6 IL-$4^{-/-}$ mice were bred in-house. All experimental procedures with mice were approved by the Institutional Animal Care and Use Committee of Washington University. Snails infected with *S. mansoni* (strain NMRI, NR-21962) were provided by the Schistosome Research Reagent Resource Center for distribution by BEI Resources, National Institute of Allergy and Infectious Diseases, National Institutes of Health. SEA was prepared from isolated schistosome eggs as previously described (16, 20). Stag was made from culture-derived *Toxoplasma gondiii tachyzoites* (Strain PTG, a clone of the ME49 line), according to a previously described method (21). Omega-1, IPSE/α, and kappa-5 were purified from SEA, as described (22, 23). For all immunizations, 30 μg extract was injected s.c. into rear footpads of mice.

In vivo treatments

For B cell–depletion experiments, beginning at 90 d post-SEA injection, BALB/c 4get/KN2 mice were injected i.p. with 50 μg anti-CD20 (a generous gift from Genentech) every 7 d for 4 wk. At the time of the fourth injection, 25 μg mAb also was injected into the footpad that had been immunized previously. For IL-4R–blockade experiments, at 60–75 d post-SEA injection, BALB/c 4get/KN2 mice were injected i.p. with 250 μg anti–IL-4R Ab (M1) at days −1 and 1 postsecondary SEA immunization. An additional 50 μg anti–IL-4R mAb was injected s.c. into the footpad at day 1 postsecondary immunization. For BrdU labeling of CD4 T cells, mice were injected i.p. with 0.5 mg BrdU (BD Biosciences or Sigma-Aldrich) and sacrificed 16 h later. For FTY720 experiments, mice were injected i.p. with 70 μg FTY720 (Cayman Chemical) every 12–14 h six times.

Flow cytometric analysis

Popliteal lymph nodes (LNs) were removed and dissociated into single-cell suspensions, as previously described (8). Surface staining with mAbs, acquisition, and analyses were performed essentially as described previously (8). Samples were acquired using a FACSCanto II flow cytometer (BD) and analyzed with FlowJo software (TreeStar). Cytokine production by T cells was determined by intracellular staining (Ab identification below) after restimulation for 5 h with PMA (50 ng/ml) and ionomycin (1 μg/ml) in the presence of brefeldin A (10 μg/ml) (24). BrdU incorporation was detected with a BrdU Flow kit (BD Pharmingen), according to the manufacturer's instructions. The following mAbs (BD, eBioscience, BioLegend, R&D Systems, or Invitrogen) against mouse Ags were used as PE, PE-Cy5, PE-Cy7, allophycocyanin, allophycocyanin-Cy7, Pacific blue, or biotin conjugates: BrdU (MoBU-1), CD4 (RM4-5), CD19 (1D3), CD138 (281-2), IgG1 (A85-1), IgD (11-26), IgM (11/41), HuCD2 (RPA-2.10), PD-1 (J43), CXCR5 (2G8), and IFN-γ (XMG1.2). IL-21R/FC chimera (R&D Systems; 596-MR-100) was used to detect IL-21–producing cells, followed by detection with PE-conjugated anti-human Ab (BD). Biotinylated Abs were secondarily stained with allophycocyanin-Cy7–conjugated streptavidin. For identification of GFP^+ fixed cells, cells were permeabilized and stained with unconjugated anti-GFP (eBioscience), washed, and stained with FITC-conjugated Goat anti-Rabbit IgG secondary Ab (Jackson ImmunoResearch). Fc-block (anti-mouse CD16/32 clone 93) was used in all experiments to minimize nonspecific signals. Plots shown are on a logicle scale.

ELISA and ELISPOT

SEA-specific serum IgG1 and end point titers were determined by ELISA using the IgG1-specific mAb X56 (BD). Immulon 4HBX plates (Thermo Fisher Scientific) were coated overnight at 4°C with 2 μg/ml SEA or Stag, blocked with 1% milk, and incubated with serial dilutions of sera, followed by a peroxidase-coupled anti-mouse IgG1 and ABTS substrate. Single Ag titers were determined as above, except that plates were coated with 500 ng/ml Ag. For ELISPOTs, single-cell bone marrow suspensions were cultured in RPMI 1640 supplemented with FCS for 24 h in MultiScreen-HA plates (Millipore, Billerica, MA) coated with 2 μg/ml SEA. Bound Ab was detected with HRP-labeled anti-mouse IgG1 (SouthernBiotech), using the AEC Chromogen Kit (Sigma-Aldrich), per the manufacturer's instructions; spots were counted using an ImmunoSpot Analyzer (v4.1; CTL).

Statistical analyses

Data were analyzed with ANOVA, followed by unpaired Student *t* test, or Pearson correlation test using Prism 6.0 (GraphPad). The p values ≤ 0.05 were considered statistically significant.

Results

IL-4 and IL-21 production is coupled in Tfh cells responding to secondary immunization with type 2 response–inducing soluble pathogen–derived Ag

We used 4get/KN2 mice, in which cells that have expressed IL-4 are marked by GFP and cells that are producing IL-4 express surface HuCD2 (25), to examine Tfh cell and GC development in popliteal LN draining sites of s.c. injection with unadjuvanted SEA. As shown previously (8), SEA immunization resulted in a robust $CD4^+CXCR5^+PD\text{-}1^+$ Tfh cell response by 8 d after primary immunization (Fig. 1A). Within this population, the majority of cells were GFP^+HuCD2^+ (Fig. 1B). The strong Tfh response was accompanied by GC B cell (defined as $CD19^+FAS^+PNA^+$), plasmablast (defined as $CD19^+CD138^+IgD^-$ cells), and plasma cell (defined as $CD19^-CD138^+IgD^-$ cells) responses (Fig. 1C).

In previously published work, Tfh cells that developed in response to immunization with a model Ag in adjuvant were found to be unexpectedly persistent in reactive LNs (19). We asked whether Tfh cells were long-lived following immunization with SEA. We were able to detect distinct Tfh cell populations in mice that were immunized with SEA 60 d earlier (Fig. 1A, data not shown). These populations were detectable in reactive LNs for prolonged periods (>180 d, data not shown). Notably, persistent Tfh cells remained active, as revealed by the fact that they continued to produce IL-4 (reflected by HuCD2 expression) (Fig. 1B). This prolonged responsiveness was associated with persisting GC B cells (Fig. 1C).

Next, we examined Tfh cell responses following secondary immunization. We found that secondary immunization resulted in marked and rapid (within 3 d) expansion of the Tfh cell pool that was apparent both in the frequency of these cells and in overall numbers (Fig. 1A, data not shown). The majority of Tfh cells were GFP^+, and approximately half of these cells were actively making IL-4 (Fig. 1B). Interestingly, secondary immunization did not result in increased GC B cell frequencies, although overall numbers increased as a result of LN expansion (this point will be addressed further below). Moreover, there was a notable increase in the frequency of plasmablasts within the B cell compartment (Fig. 1C). Plasma cell production is strongly supported by Tfh cells through the production of IL-21 (26). Consistent with previous reports about the frequency of IL-21–producing cells within the Tfh cell compartment (18), we found that IL-21 was made by ∼20% of Tfh cells in mice immunized and challenged with SEA (Fig. 1D). As might be anticipated, given the type 2 bias of the SEA-induced response, relatively few Tfh cells made IFN-γ; moreover, the

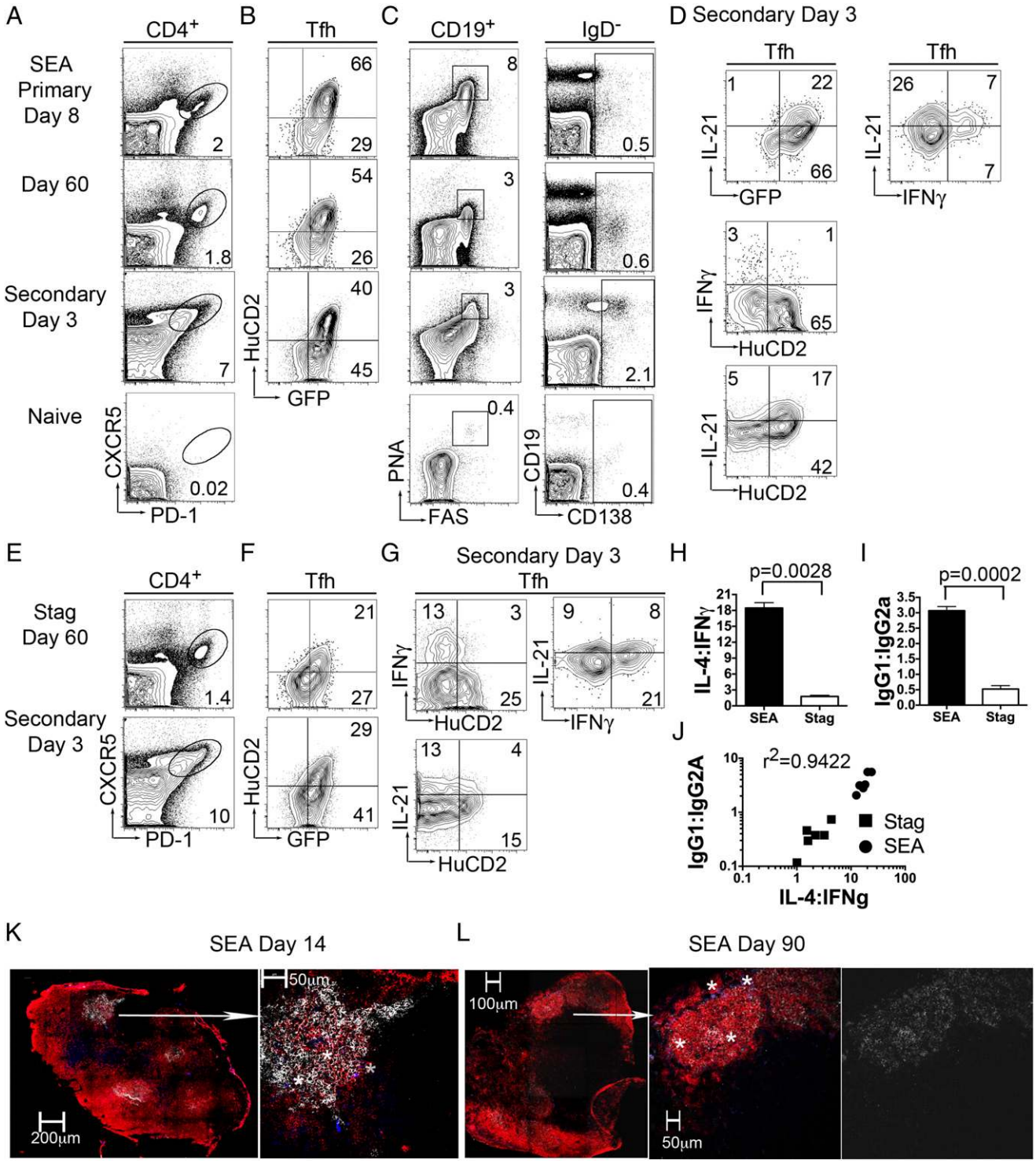

FIGURE 1. Tfh cell and B cell responses following primary or secondary immunization with SEA. BALB/c 4get/KN2 mice were immunized s.c. in the footpad with SEA, and lymphocytes were isolated from the draining popliteal LNs at days 8 and 60 postprimary immunization and at day 3 postsecondary immunization. Cells were identified by staining for the indicated markers and analyzed by flow cytometry. (**A**) Tfh cells, defined as $CD4^+CXCR5^+PD\text{-}1^+$, in immunized mice compared with naive mice. (**B**) Expression of GFP and HuCD2, as markers of IL-4 transcription and protein production, respectively, in Tfh cells, gated as in (A). (**C**) GC B cell responses, defined as $CD19^+PNA^+Fas^+$ cells, and plasmablasts and plasma cells, defined as $IgD^-CD19^+CD138^+$ and $IgD^-CD19^-CD138^+$, respectively, in immunized mice compared with naive mice. (**D**) IL-21, IFN-γ, and HuCD2 expression in Tfh cells. Cells were restimulated, as described in *Materials and Methods*, prior to staining for intracellular IL-21 and IFN-γ. (**E**–**G**) BALB/c 4get/KN2 mice were immunized s.c. in the footpad with Stag, and lymphocytes were isolated from the draining popliteal LNs at day 60 postprimary immunization and day 3 postsecondary immunization. (E) Tfh cells, defined as $CD4^+CXCR5^+PD\text{-}1^+$, in immunized mice. (F) Expression of GFP and HuCD2 in the Tfh cells gated as in (E). (G) IL-21, IFN-γ, and HuCD2 expression in Tfh cells. Cells were restimulated, as described in *Materials and Methods*, prior to staining for intracellular IL-21 and IFN-γ. (**H**) Ratio of IL-4/IFN-γ–secreting Tfh cells at day 3 postsecondary immunization. (**I**) Ratio of $IgG1/IgG2a^+$ cells in response to SEA or Stag at day 3 postsecondary injection. (**J**) Correlation between the ratio of IgG1/IgG2a and the ratio of IL-4/IFN-γ–producing cells (combined $r^2 = +0.9422$; $p = 0.0104$ for Stag, $p = 0.0478$ for SEA). FACS data are concatenated from three to five animals/group. Experiments were performed two to four times. Numbers in (A)–(G) represent frequencies of cells in quadrants or indicated gates. The Pearson correlation test and Student *t* test were used to determine statistical significance. (**K** and **L**) Frozen (*Figure legend continues*)

production of this cytokine was uncoupled from that of IL-4 (Fig. 1D). Interestingly, however, production of IL-21 was tightly coupled to IL-4, but not IFN-γ, production (Fig. 1D).

The coupling of IL-4 to IL-21 production in Tfh cells in a type 2 response raised the question of the nature of the Tfh cytokine response in the context of a type 1 response. To examine this, we immunized mice with Stag, an unadjuvanted soluble Ag from the protozoan parasite *T. gondii*. Stag has been used for studying type 1 immunity (27) and, as such, is a good counterpart to SEA for our studies. The overall intensity of the primary Tfh response, the persistence of Tfh cells following immunization, and the expansion of the response following secondary immunization were very similar to what was observed in SEA-immunized animals (Fig. 1E, data not shown). However, we were surprised to find that the frequencies of GFP^+ Tfh cells were >60% in Stag-immunized mice and that, within the GFP^+ population in Stag-immunized mice, a substantial number of Tfh cells were actually making IL-4 (Fig. 1F). Although these frequencies are lower than those elicited by SEA, they indicate that the majority of Tfh cells elicited in response to a Th1 Ag open the IL-4 locus. This finding is consistent with conclusions from other studies that, regardless of the overall bias of the immune response, IL-4 production is a signature of all Tfh cells (10, 28, 29). From previous work it is clear that IFN-γ is the major T cell cytokine associated with Stag stimulation (21), and this cytokine is critical for class switching to IgG2a/IgG2c (30, 31). Therefore, we asked whether Tfh cells from Stag-immunized mice make IFN-γ. We found a substantially higher frequency of IFN-γ^+ Tfh cells in Stag-immunized mice compared with SEA-immunized mice (Fig. 1G); in both cases there was relatively little overlap in IFN-γ and IL-4 secretion (Fig. 1D, 1G), suggesting that there are distinct Tfh cell populations that secrete either IL-4 or IFN-γ. The ratios of IL-4/IFN-γ–producing T cells in the SEA- versus Stag-immunized mice correlated strongly with the final bias in IgG1/IgG2a in the induced responses (Fig. 1J). Finally, it was notable in these studies that, in contrast to Tfh cells from SEA-immunized mice, IL-21 and IL-4 production were largely uncoupled in Tfh cells from mice immunized with Stag, whereas approximately half of the Tfh cells making IL-21 were also making IFN-γ in Stag-immunized mice (Fig. 1G).

To confirm that persisting activated Tfh cells and GC cells identified via flow cytometry were in organized GC structures, we imaged B cells and $CD4^+$ T cells in GCs on fresh-frozen sections of draining and nondraining LNs at days 14 and 80 postimmunization. At day 14 postimmunization, LNs were enlarged, contained multiple clusters of $B220^+GL7^+$ cells with infiltrating $CD4^+GL7^+$ cells, and were bordered by $CD4^+$ cells (Fig. 1K). The $CD4^+GL7^+$ cells are likely to be Tfh cells, which were reported to express GL7 in addition to PD-1 and CXCR5 (10). Late after immunization, GCs were smaller and located peripherally within the LN, but they remained organized with a ring of $CD4^+GL7^+$ Tfh cells (Fig. 1L, denoted by *) at the outer edges of the GC $B220^+GL7^+$ cells. $GL7^+$ $CD4^+$ Tfh cells also were found occasionally in the center of the GC clusters (denoted by *).

IL-4 plays critical roles in primary and secondary responses to SEA

Given the importance of IL-4 in type 2 immunity (32, 33), we focused on the role of IL-4 in the primary and secondary Tfh/B cell response. Initially, we compared the outcome of the response to SEA in wild-type (WT) and IL-$4^{-/-}$ mice. We found that the absence of IL-4 resulted in a diminished frequency of Tfh cells at day 8, the peak of the primary response (Fig. 2A), as well as a reduction in the frequency of GC B cells (Fig. 2B). Consistent with the known function of IL-4 as a class-switch factor for IgG1, there was a markedly lower frequency of Fas^+IgG1^+ GC B cells in SEA-immunized IL-$4^{-/-}$ mice than in WT mice (Fig. 2C). This was accompanied by only a small increase in the frequency of $IgG2a^+$ cells (Fig. 2C). This reduction in $IgG1^+$ B cells correlated with a reduction in the frequency of plasmablasts (Fig. 2D). Thus, as anticipated, IL-4 is essential for the primary IgG1 response to SEA, an Ag noted for its ability to induce type 2 immunity.

Next, we assessed the role of IL-4 in the secondary response to SEA. We noted that secondary immunization with SEA resulted in the appearance, within 4 d, of a population of plasmablasts in responding LNs (Fig. 2E). The kinetics of this B cell response paralleled the expansion of the IL-4–producing Tfh cell pool following secondary immunization (data not shown). To test the role of IL-4 in this process, WT mice primed with SEA 60 d earlier were given a secondary immunization, with or without blocking anti–IL-4R mAb (34). We found that anti–IL-4R blockade had no effect on Tfh cell expansion (Fig. 2F), but it substantially reduced the plasmablast pool in the draining LN, as well as in the blood (Fig. 2G, 2H). Taken together, our data indicate that IL-4 production plays a critical role in the response to SEA in IgG1 class-switching and in the expansion of the plasma cell pool that underlies the enhanced Ab response that characterizes secondary immunization.

Reciprocal Tfh cell/B cell interaction is critical for the secondary Tfh cell and plasmablast response

B cells were shown to be necessary for the generation and maintenance of the Tfh cell compartment early following primary immunization (5, 8, 35–38). In light of these reports, we evaluated the importance of B cells for the maintenance of persistent Tfh cells late after immunization, as well as for the expansion of the Tfh cell population following secondary immunization. For these experiments, mice immunized with SEA 90 d earlier were treated with anti-CD20 mAb to deplete B cells (39, 40) or isotype control Ig. This led to the rapid depletion of >95% of naive and memory B cells, although GC B cells remained in the draining LN (data not shown). We assessed $CD4^+$ T cells and B cells in the reactive LN 45 d following initiation of anti-CD20 mAb treatment. We found that the frequency of Tfh cells, the percentages of these cells that continued to express HuCD2, and the overall numbers of Th2 cells and Tfh cells were unaffected by anti-CD20 mAb treatment (Fig. 3A, 3C). However, we observed a decreased frequency of B cells (Fig. 3B) that reflected lasting effects of mAb treatment on the numbers of naive B cells and Fas^-IgG1^+ memory B cells (Fig. 3B, 3D). Because of the loss of other B cell populations, the frequency of GC B cells increased in anti-CD20 mAb–treated mice (Fig. 3B), although treated and control mice had equivalent total numbers of these cells at the time of analysis (Fig. 3D). We then gave these mice a secondary immunization and analyzed the subsequent response. We found that the expansion of Tfh cells was substantially diminished in mice that had been treated with anti-CD20 mAb and that substantially fewer cells were expressing the HuCD2 marker of IL-4 production within the Tfh population compared with control mice (Fig. 3E). Taken together, these data indicate that the emergence of an enlarged, activated Tfh population following secondary immunization is dependent on naive and/or memory B cells, but the persistence of the active

popliteal LN sections from the indicated time points were stained for B220 (red), GL7 (white), and CD4 (blue) and imaged with a laser scanning confocal microscope. Cells positive for both CD4 and GL7 appear purplish blue (*). Images are representative of three to five individual animals.

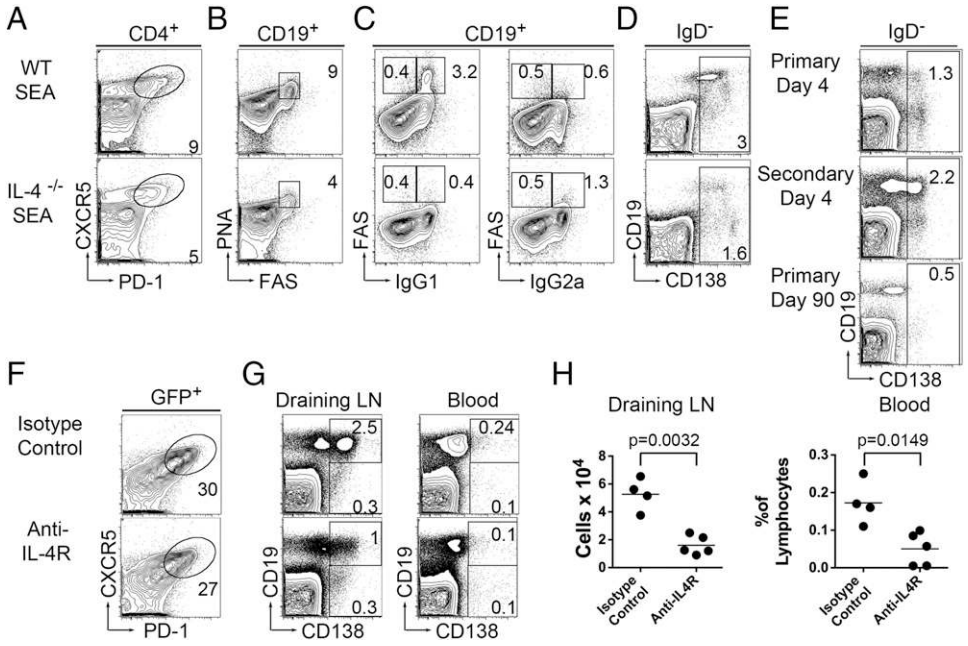

FIGURE 2. IL-4 is required for optimal Tfh cell and GC development in a primary type 2 response, as well as for the expansion of the plasmablast population that characterizes the secondary type 2 response. B6 IL-4$^{-/-}$ or WT mice (**A**–**D**) or BALB/c 4get/KN2 mice (**E**–**H**) were immunized once or twice s.c. with SEA, and lymphocytes were isolated from the draining popliteal LNs 8 or 90 d postprimary immunization (A–E) or at day 3 following secondary immunization (F–H). Cells were stained for the indicated markers and analyzed by flow cytometry. (A) Tfh cells. (B) GC B cells. (C) IgG1$^+$ and IgG2a$^+$ GC B cells. (D and E) Plasmablasts and plasma cells. (F–H) At day 90 postprimary immunization, mice were given anti–IL-4R mAb or isotype control mAb and a secondary immunization with SEA. Three days later, cells from the draining LNs or blood were analyzed by flow cytometry. (F) GFP$^+$ Tfh cells. (G) Plasmablasts and plasma cells. (H) Total numbers of plasmablasts/LN (*left panel*) and frequency of plasmablasts in the blood (*right panel*). Symbols in (H) represent individual mice. Data shown are concatenated from four or five animals/group (A–D) and five or six mice/group (E–H). Experiments in (A)–(D) were repeated twice, and those in (E)–(H) were repeated three times. Numbers in (A)–(G) represent frequencies of cells in quadrants or indicated gates. Statistical significance was determined using Student *t* test.

Tfh population for prolonged periods following primary immunization is not [rather, Tfh cell persistence is reported to depend on GC B cells (5)]. Our data indicate that naive and/or memory B cells are necessary for both the expansion of the Tfh cell compartment and for the stimulation of IL-4 production from newly differentiated Tfh cells.

Because SEA-immunized mice treated with anti-CD20 mAb displayed a significant loss of IgG1$^+$ memory B cells, but not IgG1$^+$ GC cells (Fig. 3B), we were able to examine the contributions of each of these populations to the enlarged plasmablast pool following secondary challenge (Fig. 2E). We considered it likely that the expanded population of plasmablasts that characterizes the secondary response to SEA was derived from memory B cells being recruited into the secondary response. We found that the frequencies of B cells within reacting LNs increased as a result of secondary immunization in control mice and in anti-CD20–treated mice (Fig. 3F compared with Fig. 3B); although there was a greater frequency of GC B cells, there was a reduction in both the frequency (Fig. 3F) and number (Fig. 3G) of plasmablasts in the animals treated with anti-CD20 mAb. This reduction in plasmablast numbers was accompanied by a significant reduction in anti-SEA IgG1 titers compared with control mice (Fig. 3H). Our data suggest that memory B cells are responsible for generating ≥50% of the plasmablasts that rapidly emerge in a reacting LN following secondary challenge and that this process is critical for the enhanced secondary response to SEA.

Tfh cell, Th2 cell, and plasma cell numbers expand rapidly and equivalently following secondary immunizations that target previously reactive LNs or nonreactive LNs

Our data indicate that immunization with SEA induces a persistent, active Tfh cell compartment within reactive LNs and that there is a potent secondary Tfh response following secondary immunization. We wondered whether the persistent Tfh cells contribute to the nature of a secondary immune response and began to address this experimentally by comparing secondary responses in mice immunized either in the same footpad that received the primary SEA injection to engage the persistently active popliteal LN (a "secondary" immunization, as performed in prior experiments in this study) or in the contralateral site to engage the previously nonreactive LN (a "contralateral secondary" immunization) (Fig. 4A). We found that secondary immunization led to an accelerated and marked increase in GFP$^+$ T cells that included non-Tfh cells [defined as CD4$^+$GFP$^+$CXCR5$^-$PD-1$^-$, which we previously classified as Th2 cells (8)], as well as Tfh cells in the draining LN, which substantially exceeded the magnitude of a primary response of similar duration (Fig. 4A–C). This was recapitulated, albeit at slightly reduced intensity, following contralateral secondary immunization (Fig. 4B, 4C). In both cases, the secondary T cell responses peaked at day 4 postchallenge, which preceded the day-8 peak of the primary response (Fig. 4B, 4C), and was marked by substantially increased overall numbers of IL-4–secreting cells at this time compared with at the peak of the primary response (Fig. 4D). The secondary B cell response largely mirrored the secondary T cell response, with more IgG1$^+$ class-switched B cells being generated by day 4 postchallenge than at any point during the primary response, regardless of which footpad was immunized (Fig. 4E, 4F). Overall SEA-specific IgG1 titers were indistinguishable between mice that had received secondary or contralateral secondary immunizations (Fig. 4G), a finding that was consistent with the fact that bone marrow from mice immunized in these two ways contained equivalent numbers of plasma cells, enumerated as SEA-specific IgG1 Ab-secreting cells (ASCs) (Fig. 4H). SEA-specific ASCs were undetectable in the bone marrow (or draining LN) of mice that had received a primary

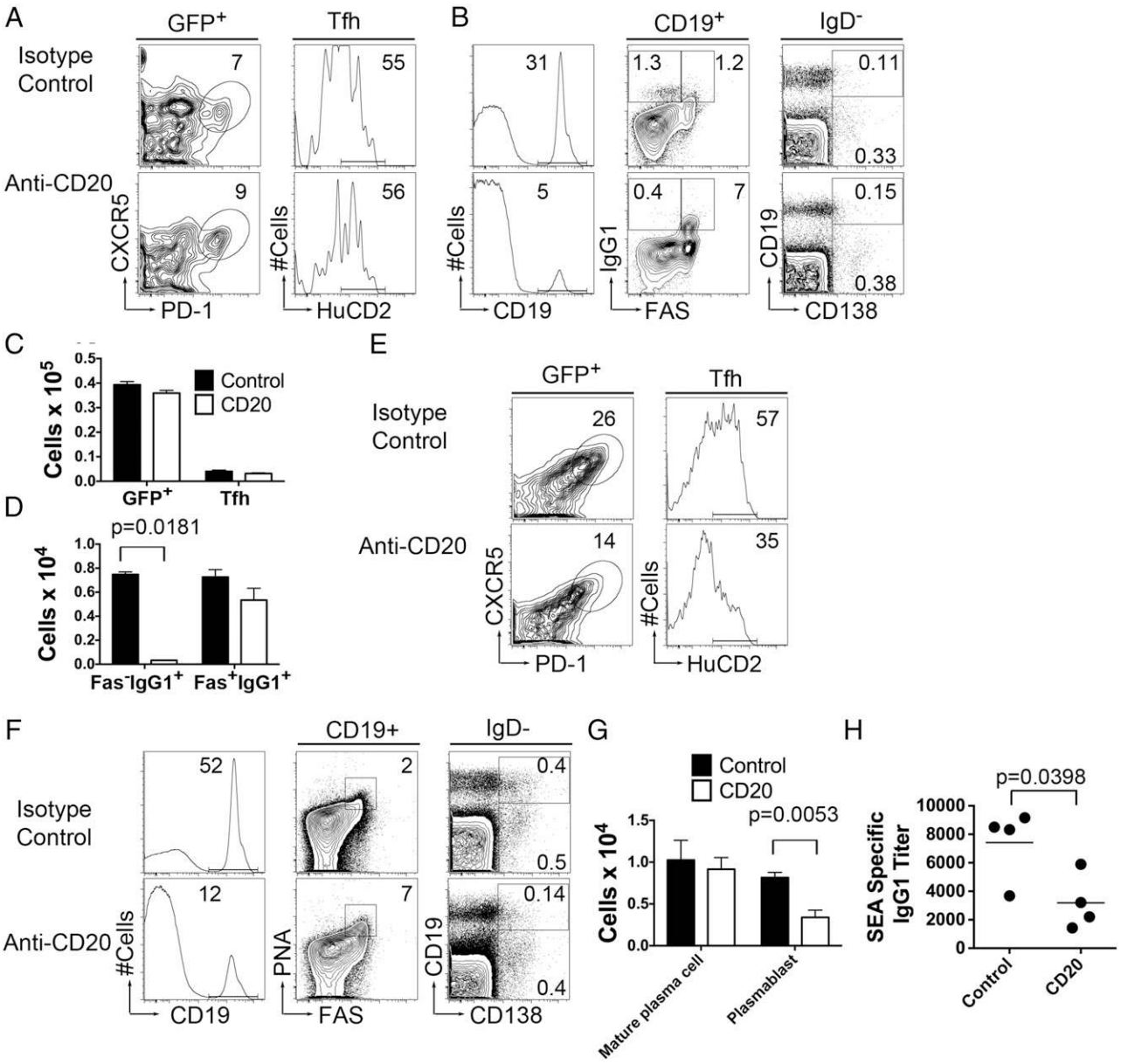

FIGURE 3. B cells are required for increases in Tfh cells, IL-4 production by Tfh cells, and numbers of plasmablasts that occur following secondary immunization. BALB/c 4get/KN2 mice were immunized with SEA and treated 90 d later with either anti-CD20 mAb or isotype control mAb. Forty-five days later, the mice were injected with PBS (**A**–**D**) or received a secondary immunization with SEA (**E**–**H**). Three days later, popliteal LN cells were isolated, stained for the indicated markers, and analyzed by flow cytometry. (A and E) GFP$^+$ Tfh cells and HuCD2 expression in gated GFP$^+$ Tfh cells. (B and F) Frequencies of B cells, Fas$^-$IgG1$^+$ memory B cells, FAS$^+$IgG1$^+$ GC B cells, plasmablasts, and plasma cells. (C and D) Total numbers of GFP$^+$ T cells, Tfh cells, FAS$^-$IgG1$^+$ memory B cells, and Fas$^+$IgG1$^+$ GC B cells at day 45 after anti-CD20 mAb treatment of mice that had received a primary immunization with SEA. (G) Total numbers of mature plasma cells and plasmablasts at day 3 postsecondary immunization in anti-CD20 mAb–treated or control mice. (H) SEA-specific IgG1 titers at day 3 postsecondary challenge of anti-CD20 mAb–treated or control mice. Symbols in (H) represent individual mice. Data in (A)–(C) are concatenated from three or four mice, and experiments were performed twice. Numbers in (A), (B), (E), and (F) represent frequencies of cells in quadrants or indicated gates. Data shown in (F)–(H) are concatenated from three to five mice/group. Data in (C), (D), and (G) are mean ± SEM. Statistical significance was determined with a Student *t* test.

immunization with SEA 4 d earlier, and they were detectable, but only in low numbers, in the bone marrow of mice immunized 90 d previously (Fig. 4H). As expected given these findings, Ab titers following secondary immunization and contralateral secondary immunization were comparable, but both were significantly higher than those measured early or late following primary immunization (Fig. 4G).

We were interested in the specific target Ag of the type 2 response induced by SEA immunization. Previous work implicated three glycoproteins as major target Ags within SEA: omega-1, IPSE/α-1, and kappa-5 (41, 42). Therefore, we used ELISA to measure Abs against these Ags and found that, although only IPSE/α-1 was strongly targeted during the primary response, all three became targets for strong IgG1 responses following secondary immunization with SEA (Fig. 4I, 4J).

The enhanced Tfh response during secondary immunization results from the recruitment of memory CD4$^+$ T cells into the Tfh pool

The conventional view of CD4$^+$ T cell responses to acute Ag exposure is that Ag-specific cells proliferate to a maximal number; thereafter, the population contracts to leave quiescent long-lived IL-7R$^+$ mem-

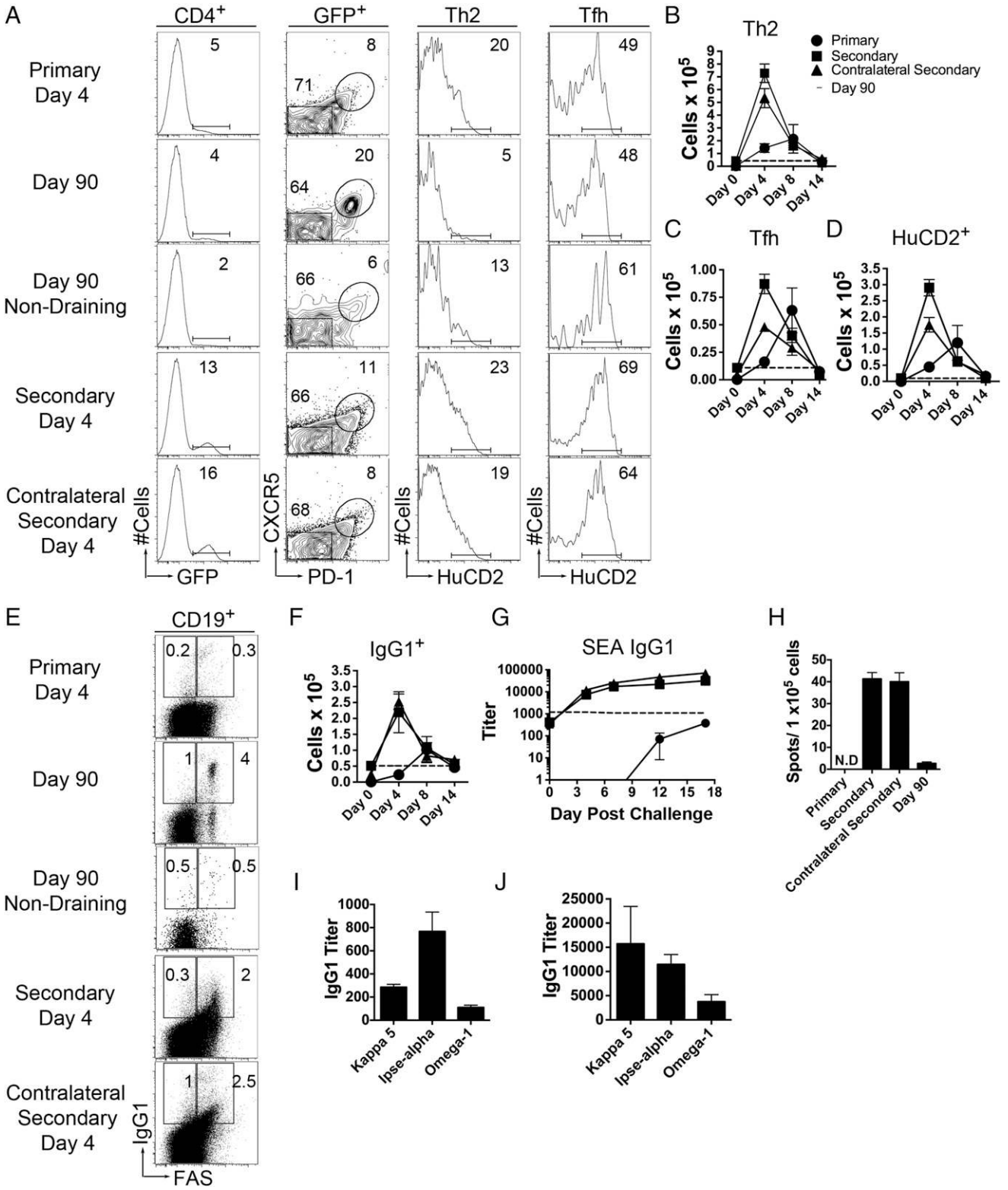

FIGURE 4. Secondary immunization induces a rapid expansion of Tfh cells, GC B cells and plasma cells, with a corresponding increase in IL-4 and Ab production. BALB/c 4get/KN2 mice were immunized once or twice with SEA and lymphocytes from reactive LNs following primary immunization (at day 8 "Primary" or Day 90), or at day 4 postsecondary immunization ("Secondary" and "Contralateral Secondary", as described in the text), were analyzed by flow cytometry for expression of the markers indicated. Mice were also bled for serum collection, and B cells in bone marrow were plated for ELISPOT detection of SEA-specific IgG1-secreting cells. (**A**) Frequencies of $CD4^+$ T cells that are competent to make IL-4 (GFP^+; *first column*); frequencies of GFP^+ Tfh cells and Th2 cells ($GFP^+PD\text{-}1^-CXCR5^-$; *second column*); and frequencies of Th2 cells and Tfh cells that are expressing HuCD2 as a marker of IL-4 production (*third* and *fourth columns* respectively). Total numbers of Th2 (**B**), Tfh (**C**) $HuCD2^+$ cells (**D**) and $IgG1^+FAS^+$ GC B cells (**F**), in draining LN of mice immunized as shown in the key for (B), at days 0 - 14 post SEA injection. (**E**) Frequencies of FAS^-IgG1^+ memory B cells and $IgG1^+FAS^+$ GC B cells in reactive LN. (**G**) Time-course of SEA specific IgG1 titers following primary and secondary SEA immunizations, as indicated in the key for (B). (**H**) Numbers of SEA-specific IgG1 secreting cells ("spots"), as measured by ELISPOT, at day 4 (Primary) or day 90 post primary immunization, or at day 4 postsecondary or contralateral secondary immunization. Serum IgG1 titers for the indicated purified SEA proteins at day 90 (*Figure legend continues*)

ory cells that are able to respond strongly upon challenge with the same Ag (43, 44). Consistent with this, in our system, where few $CD4^+$ cells are either IL-$7R^+$ or GFP^+ Tfh cells in naive LNs (Fig. 5C), the majority of $CD4^+$ T cells that responded to immunization, and that were marked by GFP expression, were IL-$7R^-$ at the peak of the primary response (Fig. 5A), whereas at later times the majority of GFP^+ cells were IL-$7R^+$ (Fig. 5B). When we focused on Tfh cells within the overall GFP^+ population, we found that, at early times postimmunization, the majority of them were $BrdU^+$ following a 16-h label, expressed HuCD2 as a marker for IL-4 production, and were IL-$7R^-$ (Fig. 5A). At later time points, the persisting Tfh cells remained $HuCD2^+$ but were no longer proliferating; moreover, they differed from the remainder of the GFP^+ population by not expressing IL-7R (Fig. 5B). Thus, Tfh cells continue to make cytokine and fail to express IL-7R, even at times when they have stopped proliferating. Interestingly, the Tfh response was largely restricted to the initially reactive LN, and we did not note any increase in Tfh cells in distal LNs, although there was an increased frequency of GFP^+ cells in these LNs compared with completely naive mice (Fig. 5C).

Next, we asked whether secondary Tfh responses differ depending on whether the challenge immunization is delivered to the same site as the primary immunization or to a distal site. Within 4 d of secondary immunization or contralateral secondary immunization, we observed an ~5–10-fold increase in the frequency of IL-$7R^-CD4^+$ T cells in reactive LNs (Fig. 6A, 6B compared with Fig. 5B, 5C). Within these populations, ~60–70% of cells incorporated BrdU within a 16-h time period; of these proliferating cells, 22–28% were Tfh cells (Fig. 6A, 6B). We reasoned that because, prior to secondary immunization, Tfh cells were IL-$7R^-$ and nonproliferative in the face of ongoing Ag presentation (which presumably underpins ongoing IL-4 production by these cells), it was unlikely that Tfh cells themselves accounted for the emergence of a proliferating population of IL-$7R^+$ Tfh cells following the additional exposure to Ag provided by secondary immunization. This led us to speculate that the secondary-response Tfh cells were derived from the proliferation and differentiation of long-lived non-Tfh $CD4^+$ T cells. The fact that Tfh cell and GC B cell numbers in LNs responding to contralateral secondary immunization were greatly increased following secondary immunization (Fig. 4C), whereas Tfh cells persisting from the primary immunization were absent (Fig. 5C), supported this view. Moreover, contralateral secondary immunization led to reactivation of $CD4^+$ cells in the originally reactive LNs and the emergence of a proliferating population of GFP^+IL-$7R^+CXCR5^+PD\text{-}1^-CD4^+$ cells (Fig. 5C) that is phenotypically consistent with reactivated central memory cells (45). We also were able to detect significant populations of these cells in draining LNs following secondary or contralateral secondary immunization (Fig. 5A, 5B). Thus, we reasoned that the recruitment of these cells into the Tfh cell pool could underpin the expansion of the Tfh cell populations observed following secondary immunization. To test this directly, we examined Tfh responses following contralateral secondary immunizations in mice treated with FTY720, which inhibits T cell egress from lymphoid organs (46–48) and, therefore, the recruitment of cells from other sites into responding LNs. At the time of peak responsiveness following contralateral secondary immunization, responding LNs in FTY720-treated mice had a higher frequency of $CD4^+$ T cells than did control mice but a reduced expansion of the $CD4^+GFP^+$ population, within which the frequency of Tfh cells was reduced 60% compared with controls (Fig. 6D); FTY720 treatment resulted in a >10-fold overall reduction in the Tfh cell population (Fig. 6E). Consistent with this, we found significantly reduced numbers of $IgG1^+$ B cells in FTY720-treated mice following contralateral secondary immunization compared with controls (Fig. 6F). We interpret these data as being consistent with the development of Tfh cells from a population of SEA-specific memory $CD4^+$ T cells recruited from the circulation into the reacting LNs during the secondary response. In this context, it is interesting that most of the cells in the Tfh population following contralateral secondary immunization were IL-$7R^+$ (Fig. 6D), which was in contrast to early or late during the primary response (Fig. 5A, 5B), and may suggest that they arose recently from IL-$7R^+$ memory cell precursors and had yet to downregulate expression of this receptor (Fig. 6C). The IL-$7R^+$ $CD4^+GFP^+CXCR5^-PD1^-$ cells evident in LN-draining sites of primary immunization (Fig. 6B), as well as in nondraining distal LNs (Fig. 6C), are candidates for memory T cells that could support this process. Taken together, these data support the view that, during secondary immunization with SEA, Tfh cells develop from a population of SEA-specific GFP^+IL-$7R^+CXCR5^+PD\text{-}1^-$ memory $CD4^+$ T cells that is recruited into the reacting LNs.

Discussion

We examined secondary responses to the unadjuvanted, soluble parasite-derived Ag SEA. We found that, in this setting, coproduction of IL-4 and IL-21 is coupled in individual Tfh cells and that activated Tfh cells persist for remarkably long periods (>60 d). Nevertheless, our data suggest that memory $CD4^+$ T cells, rather than persistent Tfh cells, are necessary for Tfh cell population expansion during a secondary response to SEA. We found that this expansion of the Tfh cell population following secondary immunization required B cells; moreover, memory B cells required IL-4 for efficient differentiation into plasmablasts, indicating that secondary type 2 immune responses depend on an intimate IL-4–centered cross-talk between B cells and Tfh cells.

Our findings support earlier work that expression of the IL-4 gene is a characteristic of Tfh cells (10, 28, 29, 49), because we observed this to be true in SEA-induced type 2 immunity, as well as in a type 1–biased response induced by Stag, a soluble extract of the parasitic protist *T. gondii*. When first reported, the fact that all Tfh cells express IL-4 was somewhat perplexing, because IL-4 is a known class-switch factor for IgG1 and IgE, but not for IgG2a or IgG2c, which are induced by IFN-γ and are characteristic of type 1 immunity. Therefore, it has been difficult to understand how production of IL-4 by all Tfh cells would be compatible with this distinction. However, we were able to show that the frequency of Tfh cells producing IL-4 in the context of type 2 immunity induced by SEA is greater than in a type 1 immune response induced by Stag, as well as that the frequency of IFN-γ–producing cells is greater in Stag-immunized mice than in SEA-immunized mice. Moreover, IL-4 production was coupled to the production of the critical Tfh cell cytokine IL-21 in SEA-immunized mice, whereas IL-4 and IL-21 production was uncoupled in Stag-immunized mice. These data support a model in which Tfh cells coordinate the production of class-switch–inducing cytokines with IL-21 to ensure that the emerging Ig isotype is functionally linked to effector functions induced in parallel by effector Th cells. The role, if any, of Tfh cell–derived IL-4 produced in type 1 immunity is under investigation in our laboratory. In addition to providing a class-switch

postprimary (**I**) and 17 postsecondary (**J**) SEA immunization. Numbers in (A) and (E) represent frequencies of cells in quadrants or indicated gates. Data shown are concatenated from three to five animals/group and all experiments were performed three times. Graphed data are mean ± SEM. N.D, not detected.

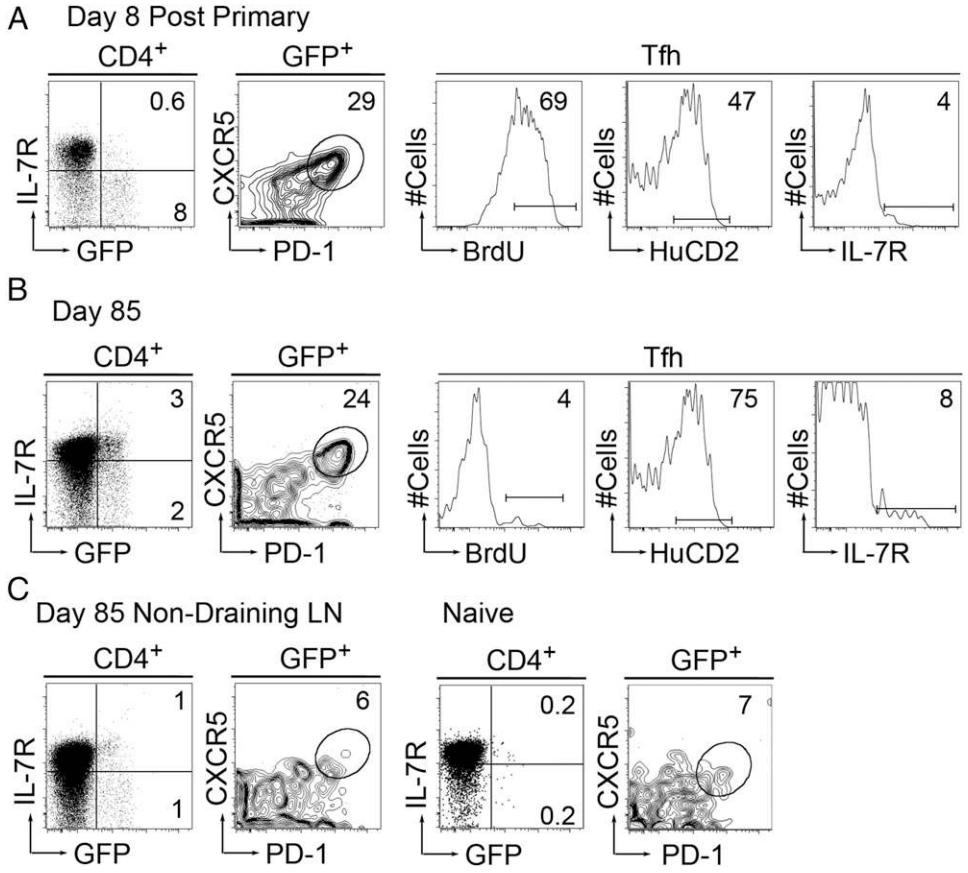

FIGURE 5. Primary Tfh cells are long-lived producers of IL-4 in the absence of proliferation or IL-7R expression. BALB/c 4get/KN2 mice were immunized with SEA and sacrificed at day 8 or 85 for analysis of cells in LNs draining sites of immunization or distal sites. Cells were analyzed by flow cytometry for expression of the markers indicated. (**A** and **B**) Expression of IL-7R in GFP^- versus GFP^+CD4^+ T cells (*first panels*), percentage of GFP^+ cells that are Tfh cells (*second panels*), BrdU incorporation in Tfh cells following a 16-h in vivo pulse (*third panels*), and HuCD2 expression in Tfh cells and anti–IL-7Rexpression in Tfh cells (*fourth* and *fifth panels* respectively), on the days indicated postprimary immunization. Data shown are concatenated from three to five animals/group, and experiments were performed three times. (**C**) Expression of IL-7R in GFP^- versus GFP^+CD4^+ T cells (*first panel*) and GFP^+ Tfh cells (*second panel*) in non-draining LNs in mice immunized for 85 d (*left two panels*) compared with naive mice (*right two panels*). Numbers represent the frequencies of cells in quadrants or indicated gates.

signal, IL-4 supports B cell survival (50); therefore, it is feasible that IL-4–producing Tfh cells are contributing to humoral responses in type 1 immunity in capacities other than Ig class switching.

Using SEA, we found that activated, $IL\text{-}7R^-$ cytokine-producing Tfh cells persist for extended periods (>60 d) following primary immunization. These Tfh cells remain associated with active GCs in which the proliferation of class-switched B cells is ongoing (data not shown). Our data show that persistent Tfh cells are discretely localized to the LNs that reacted to the primary immunization. The ability of activated Tfh cells to persist within active GCs for prolonged periods following primary immunization is a striking feature of the biology of these cells. This characteristic was reported before in the context of responses induced by adjuvanted Ag (19) and acute viral infection (17), but it has not been noted previously in responses induced by injected pathogen-derived Ag in the absence of exogenous adjuvant. We reason that prolonged GC activity reflects the capture and retention of immune complexes containing these Ags on follicular dendritic cells and that this represents the Ag depot that is sampled by GC B cells and used in cognate interactions to activate Tfh cells. The length of time over which Tfh cells and related GCs persist following immunization with SEA implies that immune complexes containing these Ag are both stable and present at high concentrations. We have little idea of the specificity of the Tfh cells or B cells that are responding to SEA. In fact, this Ag is a complex mixture of soluble molecules released when the parasite eggs are mechanically disrupted; nevertheless, three glycoproteins within this mixture—omega-1, IPSE/α-1, and kappa-5—have been defined as major Ab targets (42, 51). Of these, omega-1 has notable effects on dendritic cells and can directly affect the maturation state of these cells, conditioning them to drive type 2 responses, through its ability to interact with mannose receptor (22, 52, 53). Nevertheless, the IgG1 responses to IPSE/α-1 and kappa-5 were stronger than those against omega-1 in mice immunized with SEA, such that the titer against these Ags represented ~20% of the overall response to SEA. Based on this, we speculate that these two Ags would be found trapped in GCs in mice immunized with SEA and that Tfh cells with specificity against these Ags would be well represented within the Tfh population that develops in these animals. IPSE/α-1 was shown to possess nonspecific Ig-binding properties (54, 55), so it is feasible that it binds GC immune complexes in addition to being represented in the follicular dendritic cell Ag pool, increasing potential exposure to IPSE/α-1.

The mechanisms underlying the biology of prolonged Tfh cell responsiveness are unclear. We found that Tfh cells that persist long after primary immunization continue to produce cytokines but are no longer proliferating. A possible explanation for this is that these cells are being constrained as a result of their defining expression of PD-1, a known inhibitory receptor that is expressed on exhausted effector T cells in chronic infections and in tumors (56, 57). In

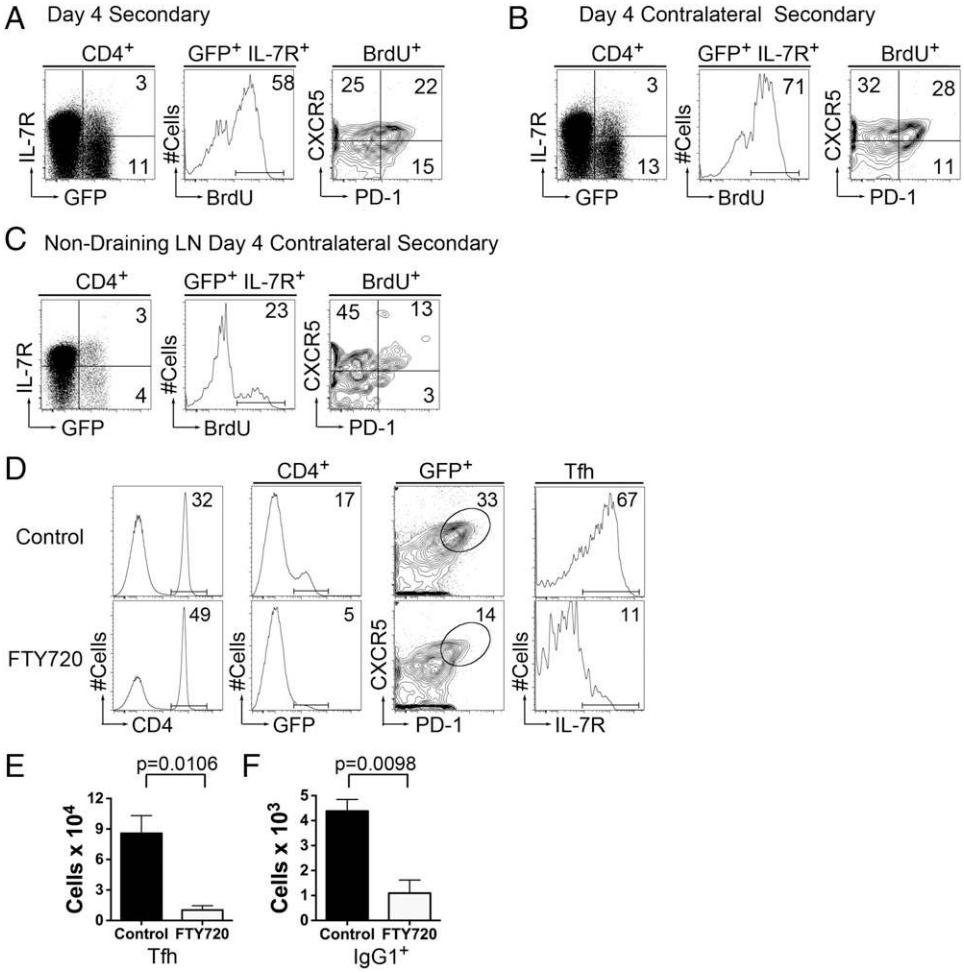

FIGURE 6. Recruitment of IL-7R$^+$ memory T cells is required for rapid and enhanced Tfh cell and plasmablast development following secondary immunization. BALB/c 4get/KN2 mice were immunized twice with SEA, and lymphocytes from the reactive or nonreactive LNs were analyzed by flow cytometry at day 4 postsecondary immunization for expression of the markers indicated. (**A**–**C**) Frequencies of CD4$^+$ T cells that coexpress GFP and IL-7R (*left panels*), frequency of BrdU incorporation in GFP$^+$IL-7R$^+$ cells (*middle panels*), and the percentages of BrdU$^+$ cells that express CXCR5 and PD-1 (*right panels*). Data shown are concatenated from three to five mice/group, and experiments were performed three times. (**D**–**F**) Mice were immunized with SEA; 85 d later they were given FTY720 or PBS (Control) prior to receiving a secondary immunization with SEA in a distal site. Lymphocytes from draining LNs were analyzed 3 d later. (D) CD4$^+$ T cells (*first column*), GFP$^+$CD4$^+$ T cells (*second column*), GFP$^+$ Tfh cells (*third column*), and IL-7R$^+$ Tfh cells (*fourth column*). Data shown are concatenated from four mice/group, and experiments were performed twice. (E and F) Total numbers of Tfh and CD19$^+$IgG1$^+$ B cells in draining LNs at day 3 postsecondary immunization in mice that received FTY720 or PBS prior to challenge. Data represent mean ± SEM of results from four mice/group. Numbers in (A)–(D) represent frequencies of cells in quadrants or indicated gates. Statistical significance was determined by the Student *t* test.

other systems, lack of PD1, or blockade of the interaction of this receptor with its ligand PD-L1, expressed by GC B cells, results in increases in the number of these cells (58, 59). The physiologic effects of this appear to be situation dependent: in some systems PD1/PD-L1 blockade results in increased Ag-specific Ab responses, whereas in others it leads to diminished plasma cell and GC responses (58–61).

Our experiments using anti-CD20 mAb to deplete B cells revealed a role for these cells in the expansion of the Tfh cell population following secondary immunization. Anti-CD20 mAb was reported to deplete GC B cells inefficiently (5); this was also our finding. Thus, our experiments did not directly address the role of GC B cells in the maintenance of Tfh cells in SEA-immunized mice. Nevertheless, based on previous work on the effects of blocking ICOS/ICOS-L or CD40/CD40L interactions on Tfh cells (5, 62), it seems reasonable to assume that intimate Tfh cell/GC B cell interactions are required for persistent GCs in SEA-immunized mice. Our experimental approach revealed that the loss of non-GC B cells, which include memory and naive B cells, following primary immunization significantly limits the rapid expansion of the Tfh cell population following secondary immunization. Thus, as during primary immunization (8, 36, 63, 64), B cells play a significant role in Tfh cell development following secondary immunization.

In previous work, BAFF was shown to enhance survival of ASCs that are generated from memory B cells during a recall response (65), and IL-6, IL-10, and IL-21 were implicated in the generation of plasma cells following primary immunization (66–71); however, the role of IL-4 in humoral immunity following secondary immunization has been unclear. Mice deficient in IL-4 signaling were shown to have reduced serum IgG1 titers during a primary immune response (72), but a role for IL-4 signaling in the control of plasmablast generation during a secondary response has not been reported previously. We consistently observed increased IL-4 secretion during secondary responses to SEA compared with primary responses, as well as a correlation between Tfh cell IL-4 secretion and the magnitude of plasmablast generation. In light of the fact that we observed a significant reduction in both blood and LN plasma cells when IL-4R signaling was blocked during the secondary

response, we believe that there is a requirement for IL-4R engagement on IgG1$^+$ memory B cells to induce differentiation into plasmablasts during a secondary response. This requirement has implications for vaccine development, because immunization strategies that maximize the development of the IL-7R$^+$ memory cells that we believe are able to differentiate rapidly into Tfh cells and secrete IL-4 during re-exposure to Ag should lead to a more robust development of Ag-specific Ig.

There has been great interest recently in the idea of committed memory Tfh cells. The available evidence indicates that Tfh cells that become disengaged from Ag stimulation have the potential to downregulate expression of Tfh markers, such as Bcl-6 and PD-1, and persist as memory cells (73). However, the fate of these memory cells remains a matter of debate. Some studies showed that these cells are capable of being recruited into effector T cell or Tfh cell pools upon secondary immunization (18, 45), whereas others indicated that they are committed to the Tfh cell lineage (17). Our experiments did not address this issue directly, but rather attempted to determine whether persisting Tfh cells play a role in the generation of an enhanced Tfh response following secondary immunization. Our data show that, as a reactive LN continues to support persistent active Tfh cells and ongoing GCs, it becomes home to a population of CD4$^+$ T cells that is GFP$^+$IL-7R$^+$CXCR5$^+$ PD-1$^-$, which begin to proliferate following secondary immunization; cells with this phenotype also can be found in LNs draining sites of contralateral secondary immunization. Whether these reflect Tfh cells that have disengaged from Ag stimulation and entered the memory pool or memory T cells of non-Tfh origins is unclear. Nevertheless, early during the development of a secondary response at distal sites, Tfh cells are IL-7R$^+$ and fail to develop in FTY720-treated mice, suggesting that they are emerging from the activation of recruited memory-like T cells, even as the original population of Tfh cells continues to engage B cells in the persisting GCs of the originally reactive LNs.

The function of persisting Tfh cells and GCs remains unclear. If the expansion of Tfh cell populations and the dramatic increases in Ab titers that accompany them can occur independently of persisting active Tfh cells, the role of these cells presumably lies in the ongoing maintenance of Ab titers, rather than in a memory response per se. It seems reasonable to conclude that Ag depots play an important role in this process by continuing to nucleate cognate Tfh cell/B cell interactions in GCs, allowing the ongoing production of plasma cells long after the initial exposure to Ag (74). Presumably, this type of response confers significant benefits in terms of maintaining Ab titers following prior exposure to Ag, which, in terms of immunity to infection, would be considered beneficial. However, these types of persistent Tfh/GC responses also may play detrimental roles in autoimmune settings in which Ab plays a pathologic role, and it is feasible that their resistance to anti-CD20 mAb explains the failure of anti-CD20–based therapies in the treatment of diseases such as systemic lupus erythematosus (75). Greater understanding of the role of persistent Tfh cells in resistance to infection or the development of autoimmunity may lead to rational approaches to promote or inhibit these responses.

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Disclosures

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