T follicular helper cells differentiate from Th2 cells in response to helminth antigens

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The relationship of T follicular helper (TFH) cells to other T helper (Th) subsets is controversial. We find that after helminth infection, or immunization with helminth antigens, reactive lymphoid organs of 4get IL-4/GFP reporter mice contain populations of IL-4/GFP-expressing CD4+ T cells that display the TFH markers CXCR5, PD-1, and ICOS. These TFH cells express the canonical TFH markers BCL6 and IL–21, but also GATA3, the master regulator of Th2 cell differentiation. Consistent with a relationship between Th2 and TFH cells, IL–4 protein production, reported by expression of huCD2 in IL–4 dual reporter (4get/KN2) mice, was a robust marker of TFH cells in LNs responding to helminth antigens. Moreover, the majority of huCD2/IL–4–producing Th cells were found within B cell follicles, consistent with their definition as TFH cells. TFH cell development after immunization failed to occur in mice lacking B cells or CD154. The relationship of TFH cells to the Th2 lineage was confirmed when TFH cells were found to develop from CXCR5−PD–1−IL–4/GFP+CD4+ T cells after their transfer into naive mice and antigen challenge in vivo.

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the causative agent of schistosomiasis, a neglected tropical disease affecting >200 million people (14). Our studies in this area have, on the basis of surface marker expression, identified subsets of Th2 cells (15), one of which is notable for its expression of PD-1, a molecule that has been reported to be expressed by TFH cells (6). Here, we investigate whether PD-1+ Th2 cells are, in fact, TFH cells. We find that in mice infected with S. mansoni, or in mice immunized with an extract of schistosome eggs, TFH cells comprise a major component of the Th cell response and express genes that would normally be considered markers of Th2 cells. We hypothesized that TFH cells and associated Th effector populations could share certain attributes and might reflect various stages of differentiation within the same lineage. Consistent with this, our data suggest TFH cells can differentiate from within the Th2 lineage, and that this process is germinal center dependent.

RESULTS AND DISCUSSION

Th2 response-inducing stimuli promote the development of PD-1+ IL-4-expressing cells in draining LNs that appear in parallel with germinal center development

In an analysis of the development of hyporesponsiveness within the Th2 cell population during chronic schistosomiasis, we previously noted the presence of a PD-1+ subset of Th2 cells that is absent in naive mice (Fig. 1 A) (15). To examine whether this population is present in other Th2-biased settings, we searched for PD-1+ IL-4/GFP+ CD4+ T cells in 4get mice immunized with schistosome eggs (the life stage of this parasite that most strongly induces the Th2 response; reference [8]), an extract thereof (serum egg antigen [SEA]), or OVA in alum. In each case, we observed distinct populations of PD-1+ IL-4/GFP+ cells, which constituted 30–60% of the overall IL-4/GFP+ cell population within responding LNs (Fig. 1 A).

Next, we asked when during the development of a Th2 response did PD-1+ CD4+ T cells first appear. For these experiments, we focused on SEA-immunized mice. A distinguishable population of SEA-induced IL-4/GFP+ CD4+ T cells was evident by 3 d after immunization (Fig. 1 B). PD-1+ IL-4/GFP+ cells were first apparent at 3 d, but the size of this population, and the levels of PD-1 expression within it, increased thereafter to reach a maximum at 14 d (Fig. 1 B). This time, we found >150,000 PD-1+ IL-4/GFP+ CD4+ T cells per draining LN. In contrast, <500 cells with these characteristics were present in popliteal LNs of naive mice.

Recent work has indicated that PD-1 is a marker for TFH cells (6). A principal function of TFH cells is their interaction with B cells to promote germinal center formation and Ig isotype switching. IgG1 and IgE are produced at high levels during helminth infections and, for S. mansoni, have, on the basis of surface marker expression, identified subsets of Th2 cells (15), one of which is notable for its expression of PD-1, a molecule that has been reported to be expressed by TFH cells (6). Here, we investigate whether the emergence of the PD-1+ IL-4/GFP+ CD4+ T cell population should parallel with germinal center development kinetics. Consistent with this, we observed distinct populations of PD-1+ IL-4/GFP+ cells in 4get mice immunized with SEA, and grew thereafter (Fig. 1, C and D). Consistent with the kinetics of the expansion of the germinal center B cell population, SEA-specific IgG1 titers developed between days 6 and 9 after immunization (Fig. 1 E).

IL-4 production is a marker for TFH cells that develop in a Th2 setting

The data shown in Fig. 1 suggested that the IL-4/GFP+ CD4+ Th2 cells that also expressed PD-1 could constitute a population of TFH cells. To explore this possibility, we made use of 4get/KN2 mice in which IL-4–competent Th2 cells are marked by GFP and IL-4–producing cells additionally express huCD2 on their surface. These animals were immunized with SEA, and 14 d later, draining LN cells were stained for PD-1 and the TFH markers CXCR5 and ICOS (2, 16). We found PD-1 and CXCR5 to be coexpressed by a distinct population of CD4+ T cells (Fig. 2 A). Within the CD4+ T cell populations, PD1+ CXCR5+ cells were ICOShi (Fig. 2 A). Remarkably, this population was also defined by the expression of huCD2, the marker of IL-4 protein production (Fig. 2 A). Reciprocal gating revealed that huCD2+ CD4+ T cells expressed the TFH markers PD-1 and CXCR5 (Fig. 2 B) and ICOS (not depicted). Thus, in mice mounting a Th2 response to helminth antigens, the great majority of CD4+ T cells that are producing IL-4 within reactive LNs express canonical markers of TFH cells. In contrast, we were unable to find IL-4-producing TFH cells in nondraining LNs (unpublished data). To further define the PD-1+ IL-4+/GFP+ CD4+ T cells, we purified them by FACS and used real time RT-PCR to assess expression of the TFH marker genes BCL-6 and IL-21 (2). We found both genes to be highly expressed in PD-1+ IL-4+/GFP+ CD4+ T cells (TFH cells) compared with PD-1− IL-4+/GFP+ CD4+ T cells (Fig. 2 C and D).

In contrast, and consistent with the expression of GFP in both populations, IL-4 transcripts were found in TFH cells and in PD-1− IL-4+/GFP+ CD4+ T cells (Fig. 2 F). Consistent with the IL-4 data, transcript levels of GATA3, which is considered the master regulator of Th2 cell differentiation (17), were comparable in TFH and PD-1− IL-4+/GFP+ CD4+ T cells from SEA-immunized mice (Fig. 2 E). Moreover, both TFH and PD-1− IL-4+/GFP+ CD4+ T cells expressed IL-5 and IL-13 (Fig. 3). In the system studied, TFH cells did not express RORγt, T-bet, or Foxp3 (Fig. S2).

Next, we used 4get/KN2 mice to examine IL-4 protein production during the development of the TFH cell population. PD-1+ and CXCR5+ CD4+ T cells appeared within 3–5 d of immunization with SEA, and by day 7–14 distinct populations of huCD2+ IL-4–producing PD-1+ and CXCR5+ CD4+ cells were apparent (Fig. 3, A and B). The majority of PD-1+ and CXCR5+ cells coexpressed huCD2 (Fig. 3, A and B), and PD-1+ and CXCR5+ were expressed by the same population of cells (not depicted). We noted that the relative size of the PD-1hi population on day 3 varied between experiments (compare Figs. 1 and 3); the significance of this is unclear at this time.

The data in Fig. 3 (A and B) indicate that IL-4 production by CD4+ T cells within reactive LNs is largely restricted to TFH cells. We next examined the phenotype of IL-4–producing cells within peripheral sites of antigen deposition.
In SEA-immunized mice, IL-4–producing CD4+ T cells are located within the B cell follicles of the draining LN. A defining feature of TFH cells is their localization to B cell follicles, which is a prerequisite for their interaction with B cells. To explore whether the huCD2+ IL-4–producing TFH cells in 4get/KN2 mice immunized with SEA are localized to B cell areas, we used immunohistochemistry on sections of LNs to identify huCD2+ cells in spatial relationship to T cell zones, B cell follicles, and germinal centers. Examining draining LNs 14 d after immunization, we found huCD2-expressing cells, which by definition are largely TFH cells in our system (Fig. 3 C), to be localized almost exclusively to B cell areas.

Together, these findings are consistent with a model in which there is a separation of labor within the Th2 lineage; cells that have differentiated into TFH cells provide help to B cells, whereas “conventional” Th2 cells traffic to peripheral tissues to exert their effector functions.

**Figure 1.** PD-1+ Th2 cells and germinal center B cells develop in response to helminth infections or immunization with Th2 response-inducing antigens/adjuvants. (A) 4get mice were infected with *S. mansoni* or immunized s.c. with the antigens indicated. Various times thereafter (see Results and Discussion), cells from reactive LNs were analyzed for PD-1 and IL-4/GFP expression. Data shown are from gated CD4+ T cells. (B) Time course of development of PD1+ IL-4/GFP+ Th cells after immunization with SEA. Data shown are from gated CD4+ T cells. (C) Kinetics of germinal center development in LN draining sites of SEA—immunization. Germinal center B cells were identified FAS+ PNA+ B220+. Data shown are from gated B220+ cells. (D) Numbers of germinal center B cells per draining LN at the times post-SEA injection indicated. (E) Endpoint titers of anti-SEA IgG1 in serum at days after infection indicated. In A–C, numbers are percentages of all gated cells that fall within quadrants/gates. These experiments were repeated at least twice, with three or more mice per experimental group. Error bars represent SEM.
follicles (Fig. 4 A), with few huCD2+ cells in T cell zones (Fig. 4 B). Moreover, most of these cells within B cell follicles were associated with germinal centers, identified by PNA staining (Fig. 4 C). These data strongly concur with those obtained by flow cytometry, in showing a close correlation between huCD2 expression/IL-4 production and TFH cell attributes, such as CXCR5/ICOS/PD-1 expression and the localization to germinal centers within B cell follicles. IL-4–competent GFP+ Th cells in SEA-immunized 4get mice were present in T cell zones and in B cell follicles throughout reactive LNs (unpublished data). With the results shown in Fig. 4, these observations suggest that Th cells that are competent to make IL-4, but not actively producing it, are not resident in B cell follicles.

TFH cells develop from within the Th2 lineage in a germinal center–dependent manner

The close association of TFH cells with germinal centers within the B cell follicle, and the lack of TFH cells in CD19−/− mice, which have B cells but fail to develop germinal centers (6), suggested that germinal centers might play a critical role in the differentiation of TFH cells. To evaluate this possibility, we analyzed B cell–deficient μMT and JHD mice, as well as mice that lack CD154, in which B cells are present but germinal centers fail to develop (18). In initial experiments, we directly immunized μMT/4get mice, and used flow cytometry to measure TFH cell development in draining LNs 14 d after sensitization. Strikingly, we were unable to detect CXCR5+ PD-1+ IL-4/GFP+ CD4+ TFH cells in immunized B cell–deficient μMT/4get animals (Fig. 5, A and B). This contrasted with the development of a robust TFH population in B cell–sufficient 4get mice (Fig. 5, A and B). The absence of TFH cells in the immunized μMT/4get mice was not the result of a failure to prime a Th response, as these animals possessed a robust population of SEA-induced CXCR5−PD-1− IL-4/GFP+ CD4+ T cells (Fig. 5, A and C). Interestingly, the overall percentages of CD4+ T cells expressing IL-4/GFP were similar in SEA-immunized 4get and μMT/4get mice, suggesting that the TFH population might derive from within the Th2 lineage in the presence of B cells. We repeated these experiments using JHD mice, again finding that TFH cells failed to emerge after immunization with

Figure 2. IL-4 is a marker for TFH cells that develop in a Th2 setting. (A) Reactive LN CD4+ Th cells from SEA-immunized 4get/KN2 mice were stained for the TFH markers CXCR5, PD-1, and ICOS, and for huCD2 (as a marker of IL-4 production). CXCR5 and PD-1 expression on gated CD4+ T cells after immunization with SEA (left). ICOS expression on CXCR5+ PD-1+ gated CD4+ Th cells versus isotype control (middle). IL-4 (huCD2) expression on CXCR5+ PD-1+ gated CD4+ Th cells versus isotype control (right). (B) Reciprocal gating of huCD2+ CD4+ T cells revealed the majority to express the TFH markers PD-1 and CXCR5. Numbers show percentages of huCD2+ CD4+ T cells that fall within the gates. (C–F) Expression of BCL6 (C), IL-21 (D), GATA3 (E), and IL-4 (F) by PD1+ GFP+ CD4+ T cells (TFH cells) PD-1− GFP− CD4+ T cells (Th2 cells) and GFP− CD4+ T cells (Naive T cells) sorted from the same LNs. Real-time RT-PCR was used to measure transcript levels for each of the genes indicated. For A, the experiment was performed three or more times, with three mice per group. For B–F, experiments were repeated twice, in each case using mRNA pooled from cells sorted from 10 mice per group.
SEA in the absence of B cells (unpublished data). We next asked whether TFH cells develop in the absence of CD154, a molecule that plays a crucial role in T cell–B cell interactions and that is necessary for germinal center development (18). We found that CXCR5+ PD-1+ CD4+ T cells were absent in SEA-immunized CD154−/− animals (Fig. 5D).

The lineage differentiation of TFH cells has been unclear. In our system, TFH cells possess canonical attributes of Th2 cells. Further, kinetic analysis of TFH cell development showed that the percentages of IL-4/GFP+ CD4+ T cells in draining LNs remained relatively constant (∼16% of total CD4+ cells) from day 7–14 after SEA immunization, whereas the contribution of PD-1+ TFH cells to this population doubled from 21–42% over the same time period (Fig. 1B), suggesting that TFH cells develop from within the PD-1− IL-4/GFP+ CD4+ T cell population. To formally address this, we FACS-purified IL-4/GFP+ CD4+ T cells that lacked the classical TFH markers CXCR5 and PD-1 (Fig. 4E) from the pooled draining LNs of Thy1.1 4get mice that had been immunized 5 d earlier with SEA. These cells were then adoptively transferred into congenic Thy1.2 recipient BALB/c mice or JHD mice, which were then immediately immunized with SEA. After 7 d, Thy1.1+ donor cells in the reactive LNs of recipient animals (Fig. 5F) were phenotyped for the expression of the TFH markers CXCR5 and PD-1. This experimental design allowed us to determine whether TFH cells differentiate from CD4+ T cells that have already expressed IL-4, the signature cytokine of Th2 cells because Thy1.1+ TFH cells implicitly must arise from the donor IL-4/GFP+ CD4+ T cells. It also allowed us to ask whether B cells are important for this transition. We found that in WT recipients, ∼20% of the detectable Thy1.1+ donor cells had become CXCR5+ PD-1+, although they continued to express GFP (Fig. 5, F and G). In contrast, donor cells were IL-4/GFP+, but remained CXCR5− PD-1− after transfer into B cell–deficient recipients (Fig. 5, F and G). Few transferred cells were found in pooled nondraining LNs, indicating that homing to the reactive LNs and conversion to TFH cells is driven by local exposure to antigen and/or inflammation (not depicted). Together, our results indicate that IL-4 competent Th cells have the capacity to differentiate into TFH cells and are consistent with a crucial role for germinal centers in TFH cell development in the context of a Th2 response.

A remarkable feature of our findings is that essentially all IL-4–producing cells in reactive LNs are localized to B cell follicles and express canonical TFH markers. Similar observations were made in mice infected with the intestinal helminth Heligmosomoides polygyrus and are reported by King and Mohrs in a report (19) on pp. 1001 of this issue. Data from King

Figure 3. TFH cells develop early in the response to SEA and localize to reactive lymphoid organs, not tissue sites of antigen deposition. (A) Time course of development of PD-1+ huCD2+ Th cells after immunization with SEA. (B) Time course of development of CXCR5+ huCD2+ Th cells after immunization with SEA. (C) Expression of PD-1 and huCD2 by gated GFP+ CD4+ T cells from the spleens or hepatic granulomas of 4get/KN2 mice that were infected with S. mansoni for 8 wk. Data shown in A and B represent gated CD4 T cells. These experiments were repeated at least twice, with three or more mice per group.
and Mohrs (19) show that TFH cells play a key role during helminth infection by producing the IL-4 that is essential for a productive B cell response. However, the observations from both studies raise questions about the IL-4/GFP+ CD4+ T cells that are found in reactive LNs outside the B cell follicles and are not producing IL-4 cytokine. The observation that these cells express Gata3, IL-4, IL-5, and IL-13 indicates that they have committed to the Th2 lineage, and we assume that they possess the potential to become either TFH cells within the LN or migrate to peripheral tissues to perform Th2 effector cell functions. Data from a study by Reinhardt et al. (20) using different infection models, which was published while this paper was under consideration, essentially support these conclusions. Nevertheless, it remains to be determined whether individual CXCR5−PD-1−IL-4/GFP+ CD4+ T cells have the potential to become either a Th2 effector cell or a TFH cell.

Despite recent work showing that TFH cells fail to express IFN-γ or IL-17 (or IL-4) in mice immunized with antigen in CFA, a protocol that drives Th1 and Th17 response development (4), our data support the view that TFH cells do not represent an independent lineage, but rather arise as a specialized subset from within the dominant Th2 lineage.

Data published as this paper was under consideration suggest that a similar relationship might exist between other types of Th cells and TFH cells because, in a setting where Th1 cells develop, IFN-γ production in reactive LNs was shown to be largely restricted to TFH cells (20).

Although our experiments suggest that a Th2 cell can become a TFH cell, they do not address whether this conversion is a final step in a linear pathway. Instead, there may be plasticity between TFH and Th2 cells, wherein different signals might regulate the reversible transition between the two states. Indeed, there is evidence from other systems that Th subset cells that might have previously been considered to be terminally differentiated do, in fact, retain plasticity in terms of which cytokines they are able to produce, and that this plasticity is heavily influenced by local environmental factors produced in response to the sensitizing antigen/infection (21, 22). Our data indicate that B cells and CD154 are necessary for cells within the Th2 lineage to acquire TFH attributes, and thus support the view developed by Haynes et al. (6) that germinal centers are critical for TFH cell development.

IL-21, which is expressed strongly by TFH cells, is crucial for TFH cell development (4, 23). IL-21 has also been reported to be produced by, and important for the development of, Th2 cells (24, 25). Indeed, a recent study showed a role for IL-21R in the full expression of the Th2 cell response in schistosomiasis (26). However, a distinction was not drawn between Th2 and TFH cells in these reports, and in light of the data presented here, it seems that at least some of the observations made previously could reflect analyses of mixed Th2/TFH populations. We have a similar concern about our previous interpretation of the finding that CD154 is essential for mice to survive infection with S. mansoni (27). We attributed this increased susceptibility to reduced Th2 responses. However, reinterpretation of the data in the context of the observations reported here raises the possibility of an important role for TFH cells in the prevention of the development of severe disease after infection with this parasite. Ongoing studies are addressing this issue.

The diseases caused by helminths are often chronic and debilitating, and at this time the only recourse available is chemotherapy, which must be delivered repeatedly. Vaccination is arguably the best solution to eradicate helminthiasis and other infectious diseases. Studies designed to increase understanding of the origin, development, and function of TFH cells should provide insights into the optimal requirements for antibody production, and as such, hold promise for accelerating developments in this vital area.

**Figure 4.** In SEA-immunized mice, IL-4-producing CD4+ T cells localize to the B cell follicles of reactive LNs. Sections of reactive popliteal LNs from SEA-immunized mice. B cell follicles (A); T cell zones (B). For A and B, B220 staining is shown in green, CD4 in blue, and huCD2 in red. (C) Germinal centers, with PNA in green, CD4 in blue, and huCD2 in red. Cells double positive for CD4 and huCD2 staining appear as magenta. This experiment was repeated twice. Bar, 50 µm.
BRIEF DEFINITIVE REPORT

PE-Cy7, allophycocyanin (APC), APC-Cy7, Pacific blue, or biotin conjugates: CD4 (RM4-5), CD45R (B220; RA3-6B2), CD90.2 (Thy1.2; 53–2.1), CD90.1 (Thy1.1; Ox-7), CD8a (53–6.7), CD278 (ICOS; 7E.17G9), CD279 (PD-1; J43), CD95 (Fas; Jo2), CXCR5 (2G8), and huCD2 (RPA-2.10). Biotinylated antibodies were secondarily stained with PerCP-Cy5.5– or APC-conjugated streptavidin. Biotin-conjugated PNA was obtained from Vector Laboratories. Plots shown are on a Logicle scale.

ELISA. SEA-specific serum IgG1 endpoint 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 0.5 µg SEA/well, blocked with FBS, and incubated with serial dilutions of sera, followed by a peroxidase-coupled anti-IgG1 conjugate and SureBlue TMB substrate (KPL). Serum was collected 14 d after immunization with SEA.

Cell sorting and adoptive transfer. Popliteal LN cells were pooled 5 d after immunization with SEA and FACSAria or FACSDiva cell sorters (BD) were used to purify CD4+ T cells based on the expression of particular markers, as described in the text. Sorted cells were routinely >97% pure.

Figure 5. TFH cells develop from within the Th2 lineage through a process that does not occur in the absence of B cells or CD154. (A) B6 4get or µMT/4get mice were immunized s.c. with SEA; 14 d later, draining LN cells were stained for PD-1. PD-1 staining versus GFP fluorescence on gated CD4+ T cells is shown. Numbers are percentages of cells in each quadrant. (B) Percentages of draining LN cells that are TFH cells in 4get mice and in µMT/4get mice at 14 d after immunization. (C) Percentages of draining LN cells that GFP+ CD4+ are in 4get mice and in µMT/4get mice at 14 d after immunization. (D) B6 or CD154−/− mice were immunized s.c. with SEA; 14 d later, draining LN cells were stained for PD-1 and CXCR5. Data are from gated CD4+ T cells. Numbers are percentages of cells in each quadrant. (E) Th2 cells (CD4+/GFP+/PD-1−/CXCR5−) were FACS-purified from the draining LNs of 4get Thy1.1 mice that had been immunized s.c. with SEA 5 d previously. (F) Sorted Th2 cells were transferred into Thy1.2 WT or JHd mice, which were then immediately immunized s.c. with SEA. 7 d later, draining LNs were removed and cells were analyzed by flow cytometry for CD4, Thy1.1, and GFP expression (sample plot shown on left; numbers are percentages of gated CD4+ T cells). Gated Thy1.1+ donor cells recovered from WT (middle) or JHd (right) recipients stained for the TFH markers PD-1 and CXCR5 (numbers are percentages of Thy1.1+GFP+ cell that fall within the gates). (G) The percentages of transferred Th2 cells that developed into TFH cells in the presence (WT) or absence (JHd) of B cells. In bar graphs, data are means of data from three or more mice ± SEM. For A–C, the experiment was performed once with five mice. For D–G, experiments were repeated at least twice, with four or more mice per experimental group. Error bars represent the SEM.

Flow cytometry. Cells were isolated from the mesenteric LNs or hepatic granulomas of infected mice, or from the popliteal LNs of s.c. immunized mice, as previously described (15). Mice were immunized s.c. in a rear footpad with eggs (2,500 per site) or with SEA (50 µg/site). OVA/alum immunization was performed in the same manner, using 130 µg of sterile egg white plus 1 mg of alum in a total volume of 25 µl per site. All experimental procedures with mice were approved by the Institutional Animal Care and Use Committees of the Trudeau Institute and of the University of Pennsylvania.
FACS-purified cells (~2 × 10^6) were injected i.v. into congenic animals, which were then immediately immunized with SEA into a hind footpad. 7 d later, reactive popliteal LNs were harvested for analysis by flow cytometry, as described above. Files generated from replicate mice were concatenated within respective groups for display.

**Real-time RT-PCR.** RNA was isolated using RNeasy (QIAGEN), treated with TURBO DNA-Free (Ambion), and used to synthesize cDNAs using Oligo(dT) (Promega) and Superscript II polymerase (Invitrogen). Real-time RT-PCR analysis was performed using SYBR-green (Applied Biosystems) on an ABI 7500 Fast Real-time PCR system (Applied Biosystems). Relative expression was calculated using the 2−ΔΔCt method normalized to HPRT. Dissociation curves were generated to verify the presence of a single amplicon. QIAGEN Real-time primers were used for IL-4, IL-5, IL-13, IL-21, IL-6, T-bet, Rorγt, and Foxp3. GATA-3 and HPRT primer sequences are available upon request.

**Immunohistochemistry.** Popliteal LNs were harvested from SEA-immunized animals and immediately frozen in OPT (OCT, Sigma) embedding compound (Sakura Finetek) over liquid nitrogen. Frozen LNs were cut into 8-μm sections on a Leica cryostat and fixed in a mixture of ice-cold 75% acetone/25% ethanol for 5 min. Sections were blocked in PBS plus 2% BSA and 2% normal mouse serum for 60 min, followed by avidin/biotin blocking solution (Vector Laboratories). Sections were stained with rat anti–mouse B220-Alexa Fluor 488 (clone RA3-6B2) or Lectin PNA-Alexa Fluor 568 (Invitrogen). A single amplicon. QIAGEN Real-time primers were used for IL-4, IL-5, IL-13, IL-21, IL-6, T-bet, Rorγt, and Foxp3. GATA-3 and HPRT primer sequences are available upon request.

**Online supplemental material.** Fig. S1 shows the results of real-time PCR analyses to measure IL-5 and IL-13 transcripts. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20090303/DC1.


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SUPPLEMENTAL MATERIAL

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Figure S1. TFH (PD-1+ GFP+ CD4+ T cells) and Th2 cells (PD-1− GFP+ CD4+ T cells) express IL-5 and IL-13. Real time RT-PCR was used to measure transcripts in naive CD4+ T cells, or sorted Th2 cells or TFH cells from SEA-injected 4get mice. Naive represents PD-1− GFP+ CD4+ T cells sorted from the same LNs. These data are from one experiment using mRNA pooled from 10 mice.

Figure S2. TFH cells do not express Foxp3, RORyt, or Tbet. Real time RT-PCR was used to measure specific transcripts in naive CD4+ T cells (PD-1− GFP− CD4+ T cells) or sorted Th2 cells (PD-1− GFP+ CD4+ T cells) or TFH cells (PD-1+ GFP+ CD4+ T cells) from SEA-injected 4get mice. A positive control for Tbet was provided by mRNA collected from CD4 T cells from mice immunized with Toxoplasma gondii. These data are from one experiment using mRNA pooled from 10 mice.