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IL-4–producing CD4+ T cells in reactive lymph nodes during helminth infection are T follicular helper cells

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Interleukin (IL)-4 is the quintessential T helper type 2 (Th2) cytokine produced by CD4+ T cells in response to helminth infection. IL-4 not only promotes the differentiation of Th2 cells but is also critical for immunoglobulin (Ig) G1 and IgE isotype-switched antibody responses. Despite the IL-4–mediated link between Th2 cells and B lymphocytes, the location of IL-4–producing T cells in the lymph nodes is currently unclear. Using IL-4 dual reporter mice, we examined the Th2 response and IL-4 production in the draining mesenteric lymph nodes during infection with the enteric nematode *Heligmosomoides polygyrus*. We show that although IL-4–competent Th2 cells are found throughout the B and T cell areas, IL-4–producing Th2 cells are restricted to the B cell follicles and associate with germinal centers. Consistent with their localization, IL-4 producers express high levels of CXCR5, ICOS, PD-1, IL-21, and BCL-6, a phenotype characteristic of T follicular helper (Tfh) cells. Although IL-4 was dispensable for the generation of Th2 and Tfh cells, its deletion resulted in defective B cell expansion and maturation. Our report reveals the compartmentalization of Th2 priming and IL-4 production in the lymph nodes during infection, and identifies Tfh cells as the dominant source of IL-4 in vivo.

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IL-4 is widely recognized as the canonical marker for Th2-polarized CD4+ T cells (1). IL-4 was originally identified by its function as a B cell–stimulating factor (2, 3), and numerous in vivo models of infection concur that IL-4 is critical for the isotype switch of B cells to IgE and IgG1 (4–10). IL-4 also promotes the Th2 polarization of naive CD4+ T cells in vitro (11, 12). However, in vivo studies have found that Th2 cells can develop in mice deficient for IL-4, the IL-4Rα chain, or the IL-4R–associated Stat 6 (4–8). Collectively, these observations suggest that IL-4 production in sentinel lymph nodes draining a site of infection may be more important to support type 2 B cell responses rather than to establish the underlying Th2 response. Therefore, it stands to reason that the production of IL-4 by Th2 cells should preferentially occur in B cell follicles to optimize B cell help. However, the localization and characterization of IL-4–producing T cells in the lymph nodes has not been assessed.

T follicular helper (Tfh) cells are a subset of CD4+ T cells that migrate to B cell follicles after activation and promote germinal center formation and B cell Ig isotype switching in mice and humans (13–16). Phenotypically, Tfh cells are characterized by expression of CXCR5 (an obligatory receptor for the follicle-homing chemokine CXCL13) (17), the inducible co-stimulatory receptor ICOS (18, 19), and the inhibitory receptors PD-1 (also known as CD279) (14) and B and T lymphocyte attenuator (BTLA) (13). A previous study (13) using model antigens in adjuvant have suggested that Tfh cells are a distinct lineage of T helper cells that arises independently of Th1, Th2, and Th17 effector subsets. To date, however, no studies have examined the Tfh response during infection with a Th2-polarizing pathogen. To unambiguously identify and localize Th2 cells and IL-4 production in the reactive lymph nodes in vivo, we infected IL-4 dual reporter mice (20), in which cells that express IL-4 are marked by GFP and IL-4–producing subsets additionally display surface huCD2, with the mouse gastrointestinal helminth parasite *Heligmosomoides polygyrus*. We find that IL-4–producing

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Th2 cells localize to the B cell follicles and exhibit a Tfh cell phenotype in the draining lymph nodes of infected animals. In addition, we find that although IL-4 is dispensable for the generation of Tfh cells, it is critical for a mature B cell response. To our knowledge, this is the first report to show the location of IL-4–producing T cells in the reactive lymph nodes after helminth infection. Furthermore, our results challenge the prevailing concept that Tfh cells are a distinct T helper lineage. Rather, we suggest that Tfh cells act to support the demands of the impending immune response.

RESULTS AND DISCUSSION

IL-4–producing Th2 cells are restricted to the B cell follicle and associate with germinal centers

Using direct ex vivo analysis of IL-4 dual reporter (4get/KN2) mice, we have previously shown that IL-4–producing/huCD2$^+$ cells represent only a subset of IL-4–expressing/GFP$^+$ Th2 cells in the draining mesenteric lymph nodes (mesLNs) of *H. polygyrus*–infected mice (20). Although IL-4–producing Th2 cells displayed some phenotypic and functional differences compared with GFP$^+$huCD2$^-$ cells, we did not assess whether they additionally differed with respect to their localization within the lymph nodes. To explore this issue, we infected 4get/KN2 mice with *H. polygyrus* and 2 wk later performed immunohistochemistry on mesLN sections to identify huCD2$^+$ cells in spatial relationship to T cell zones, B cell follicles, and germinal centers. As shown in Fig. 1 (A and B), CD4$^+$huCD2$^+$ cells were almost exclusively found in B cell follicles, whereas they were rarely detected in T cell areas. Additional staining with peanut agglutinin (PNA) revealed that CD4$^+$huCD2$^+$ cells were generally associated with germinal centers within the B cell follicle (Fig. 1 C). In contrast, CD4$^+$GFP$^+$ T cells that had initiated the expression of IL-4 (5) were detected throughout the lymph nodes and were not, unlike huCD2$^+$ cells, preferentially found in the B cell follicle (Fig. 1, D and E). These data show that the huCD2$^+$/IL-4–producing subset within the GFP$^+$ Th2 population is highly enriched in B cell follicles and germinal centers.

IL-4–producing Th2 cells in the reactive lymph nodes are Tfh cells

The selective localization of huCD2$^+$/IL-4–producing CD4$^+$ T cells to B cell areas suggested that they might function as Tfh cells. Indeed, flow cytometric analysis revealed that huCD2$^+$/IL-4–producing Th2 cells express high levels of several Tfh-associated markers, including CXCR5, ICOS, PD-1, and BTLA, as compared with GFP$^+$huCD2$^-$ Th2 cells or the GFP$^-$ population (Fig. 2 B; subset gating based on Fig. 2 A). Consistent with the generally activated phenotype of Tfh cells (13), we have previously shown that huCD2$^+$ cells are also CD69^{hi}, CD95^{hi}, CD62L^{lo}, and CCR7^{lo} (20, 21). Next, we assessed the expression of the Tfh-associated genes IL-21 and BCL-6 (13, 22) in sort-purified CD4$^+$ T cells that were positive for both GFP and huCD2, and compared them with GFP$^+$ cells that did not stain for huCD2. As shown in Fig. 2 C, IL-21 and BCL-6 were highly expressed in GFP$^+$huCD2$^+$ cells compared with GFP$^+$huCD2$^-$ CD4$^+$ T cells. As previously reported, both

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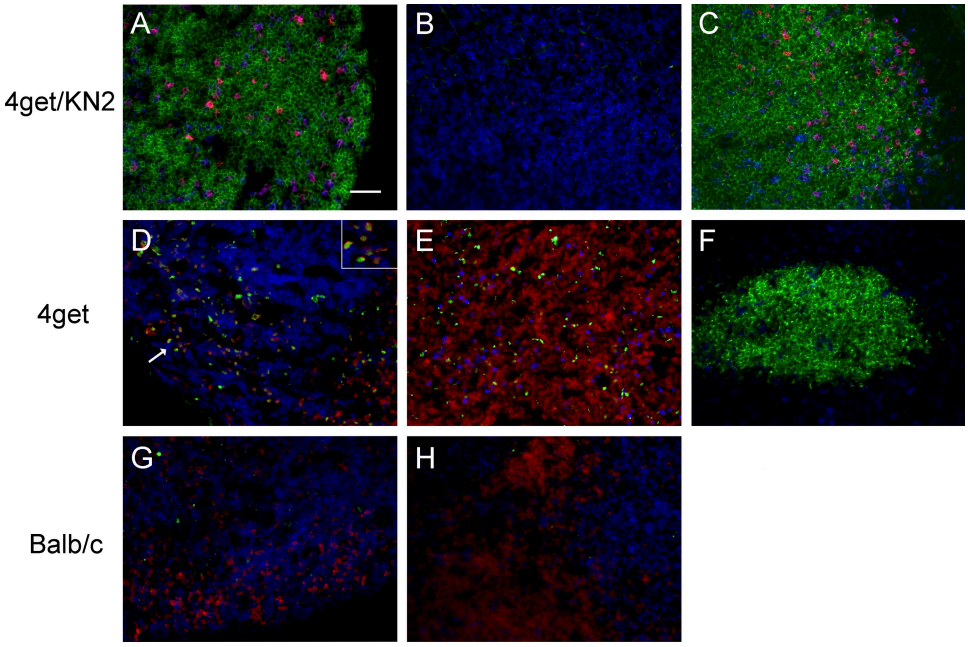

Figure 1. huCD2$^+$ IL-4–producing cells accumulate in B cell follicles and germinal centers during *H. polygyrus* infection. mesLNs from (A–C) 4get/KN2, (D–F) 4get, or (G and H) BALB/c mice at day 14 after infection with *H. polygyrus*. mesLNs in A–C are stained with CD4 (blue), huCD2 (red), and B220 (green; A and B) or PNA (green; C) for germinal center detection. F shows a huCD2 staining control for C. D, E, G, and H show cells expressing GFP (green), B220 (blue), and CD4 (red). The inset in D is an enlarged image of the area designated by the arrow. Each panel is representative of at least three separate experiments with three mice per group. Bar, 50 µm.

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GFP$^+$CD4$^+$ T cell subsets expressed IL-4 mRNA, but transcript levels were considerably higher in GFP$^+$huCD2$^+$ cells (20). Inverse gating on CD4$^+$ T cells that expressed high levels of both of the Tfh markers CXCR5 and PD-1 (Fig. 2 D) showed that essentially all of these cells also displayed huCD2 and thus produced IL-4 in situ (Fig. 2 E). Finally, gating on CD4$^+$huCD2$^+$ cells indicated that the vast majority were double positive for CXCR5 and PD-1 (Fig. 2 F). In keeping with their localization to the B cell areas of the mesLNs, these data show that IL-4–producing CD4$^+$ T cells display a Tfh cell phenotype after infection with *H. polygyrus*.

IL-4 production is restricted to Tfh cells from the onset of a Th2 response

Thus far, our experiments suggested by multiple criteria that CD4$^+$huCD2$^+$ T cells in the reactive lymph nodes represent Tfh cells in a fully established Th2 response. However, given that IL-4 is a potent promoter of Th2 cell differentiation (1, 10–12), we reasoned that IL-4 production may occur early after infection in the T cell areas of the draining lymph nodes, the site of initial priming of naive T cells by dendritic cells (23). To explore this possibility, we analyzed the mesLNs of 4get/KN2 mice on days 3–7 after infection with *H. polygyrus*. As shown in Fig. 3, CD4$^+$huCD2$^+$ T cells were, at all time points examined, preferentially found in B cell follicles, whereas they were almost completely absent from T cell zones. The few huCD2$^+$ T cells that were transiently detected in the T cell zones on day 5 appeared in close proximity to the B cell follicle (13). The inability to detect IL-4 at the presumptive site of initial antigen encounter of naive T cells is consistent with the finding that IL-4 is dispensable for Th2 priming (4–8) and suggests instead that IL-4 may play a larger role in governing the B cell response.

Tfh cells can develop in the absence of IL-4

Previous studies have suggested that IL-4 acts in an autocrine/paracrine manner to promote Th2 cell differentiation (9, 10). With this in mind, the observation that most, if not all, CD4$^+$ cells in the B cell follicles of *H. polygyrus*–infected 4get/KN2 mice produce IL-4 raises the question of whether this cytokine is required for the development of Tfh cells. Moreover, if the development of Tfh cells is dependent on IL-4, this would likely contribute to the defective antibody response observed in IL-4–deficient animals (9, 10). To determine whether Tfh cells develop in the absence of IL-4, we infected homozygous KN2 mice, which are deficient for IL-4 (20), as well as 4get/KN2 and 4get/4get control animals and analyzed the draining mesLNs 2 wk later. As shown in Fig. 4 A, the frequency of CXCR5$^+$PD-1$^+$ cells in KN2/KN2 mice was comparable to both control groups, demonstrating that Tfh cells can develop in the absence of IL-4. Consistent with our finding that huCD2 expression is a reliable marker of Tfh cells in our model (Fig. 2), CXCR5$^+$PD-1$^+$ cells were also huCD2$^+$ in IL-4–deficient KN2/KN2 animals (Fig. 4 B). Immunohistochemical analysis of draining lymph nodes from *H. polygyrus*–infected 4get/KN2 and KN2/KN2 mice revealed that CD4$^+$huCD2$^+$ cells localized normally to the B cell follicles in IL-4–deficient animals (Fig. 4 C). Collectively, these data show that the generation and follicular accumulation of Tfh cells can occur in the absence of IL-4.

Multiple B cell defects in the absence of IL-4 signals

Because the development of Tfh cells can occur in the absence of IL-4, we investigated whether local IL-4–mediated

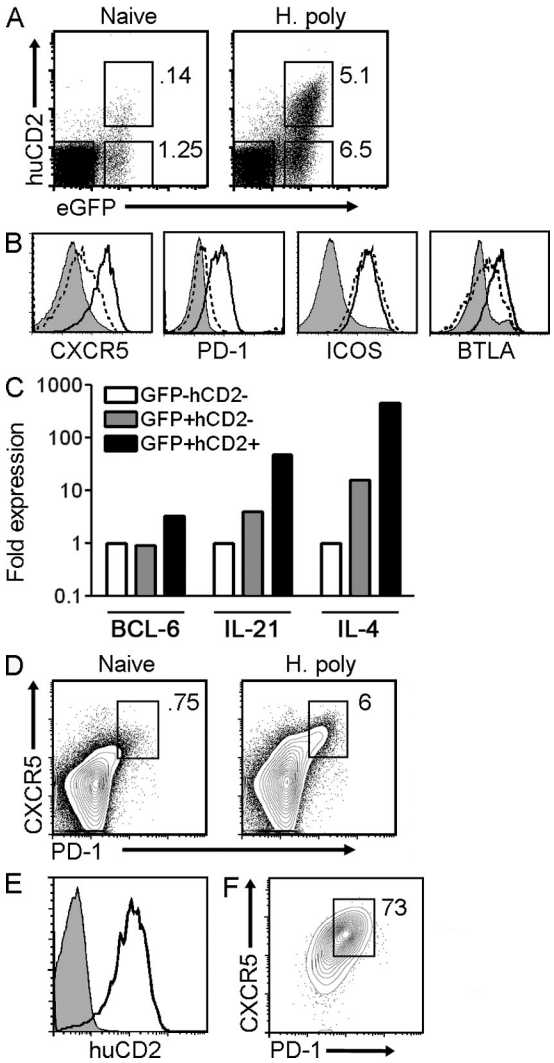

Figure 2. huCD2 marks T follicular cells in the reactive lymph nodes during helminth infection. (A) Gated CD4$^+$ T cells from mesLNs of naive or *H. polygyrus*–infected 4get/KN2 mice were assessed for enhanced GFP (eGFP) and huCD2 expression. Numbers shown indicate cell frequency within the total CD4$^+$ population. (B) CD4$^+$ subset expression of various Tfh markers. Subsets are based on the gating in A. Shaded histograms represent GFP$^-$huCD2$^-$ cells. GFP$^+$huCD2$^-$ and GFP$^+$huCD2$^+$ cells are shown as dashed and continuous lines, respectively. (C) Real-time RT-PCR analysis of the T cell subsets shown in A. Data shown are normalized to *GAPDH*. (D) The frequency of Tfh cells in naive or infected 4get/KN2 mice based on CXCR5 and PD-1 expression. (E) huCD2 expression on the mesLN CXCR5$^+$PD-1$^+$ cells shown in C. (F) CXCR5 and PD-1 expression on huCD2$^+$ cells from *H. polygyrus*–infected animals as shown in A (right). Data shown are representative of four separate experiments with three to five mice per group.

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signals in the B cell follicle affect the B cell response after *H. polygyrus* infection. To assess the specific role of IL-4 in the maturation of the Th2-associated B cell response, we infected 4get mice and IL-4Rα–deficient 4get mice with *H. polygyrus* and characterized B cell maturation and the germinal center response in the mesLNs. Of note, because T and B cells do not respond to IL-13 (24, 25), IL-4Rα–deficient mice functionally recapitulate IL-4–deficient animals with respect to these subsets while allowing for analysis of the IL-4/GFP response. Consistent with Fig. 4 and previous studies (4, 5), IL-4R$\alpha^{-/-}$ mice mounted a robust Th2 response within the first week of infection, as indicated by the substantial increase in the number of $CD4^+GFP^+$ cells (Fig. 5 A). Interestingly, this Th2 response was not maintained during the second week of infection in the absence of IL-4R–mediated functions. The analysis of the accompanying B cell response revealed that although the number of B cells in naive mice was comparable between WT and IL-4R$\alpha^{-/-}$ animals, it was strikingly reduced in IL-4R$\alpha^{-/-}$ mice 2 wk after infection (Fig. 5 B). Notably, this defect was not apparent 1 wk after infection, a time point at which Tfh cells begin to differentiate and populate the B cell follicle (Fig. 3). Moreover, the B cell population in IL-4Rα–deficient animals was significantly impaired in the up-regulation of MHC class II (I-A^d), the co-stimulatory molecule CD86, and the low affinity IgE receptor, CD23 (Fig. 5 C). Despite the presence of Tfh cells in the absence of

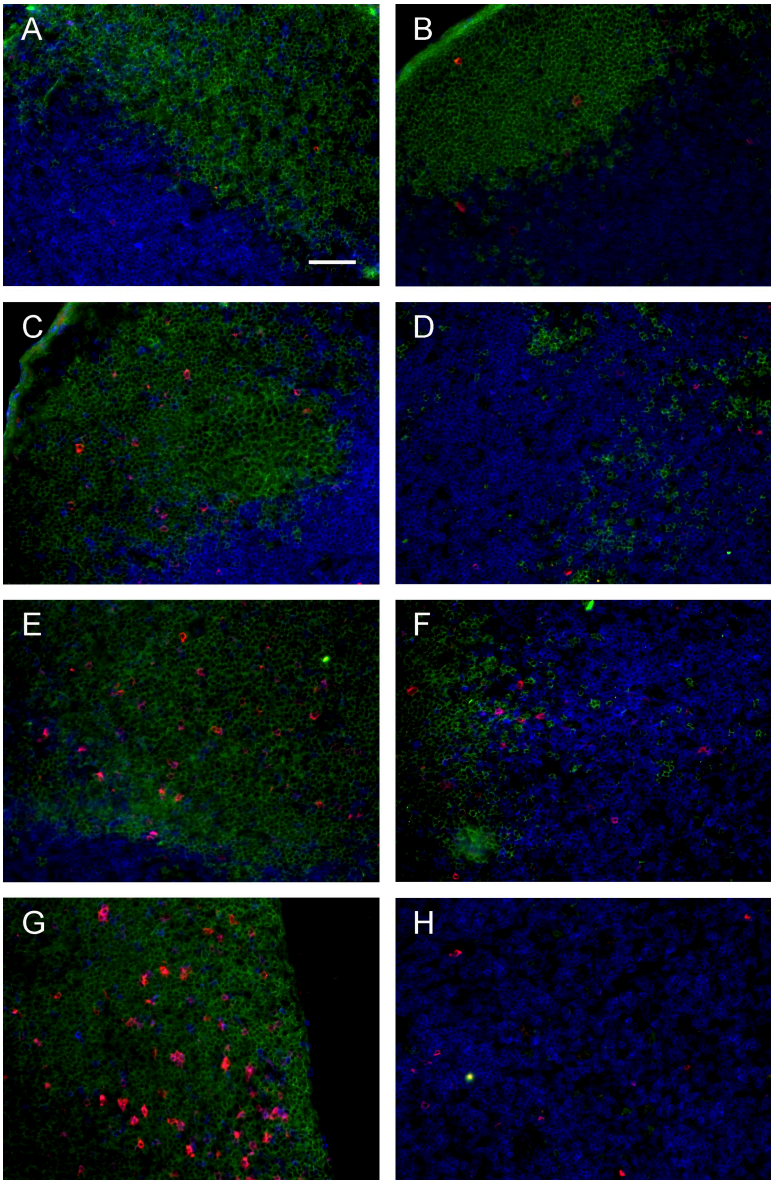

Figure 3. IL-4 producers are absent from the T cell zones during early *H. polygyrus* infection. mesLNs from (A) naive 4get/KN2 mice or at (B) day 3, (C and D) day 4, (E and F) day 5, and (G and H) day 7 after *H. polygyrus* infection. B220 (green), CD4 (blue), and hCD2 (red) stainings are shown. Each panel is representative of two separate experiments with four mice per group. Bar, 50 μm.

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IL-4 function (Fig. 4 A), there was a striking defect in the formation of germinal centers as identified by staining of CD19$^+$ B cells for PNA and Fas (Fig. 5 D). Lastly, consistent with the severely compromised IgG1 and IgE antibody response previously reported in IL-4Rα– and IL-4–deficient mice (unpublished data) (6, 7, 9, 10, 25), the frequency of IgG1$^+$/IgD$^-$ B cells was substantially decreased (Fig. 5 E) and serum IgE was undetectable (not depicted). These data show that IL-4–mediated functions, but not the mere presence of Tfh cells per se, are critical to orchestrate multiple features of an appropriately matured B cell response after helminth infection.

Our study with IL-4 reporter mice has determined the localization of IL-4–producing Th2 cells in the reactive lymph nodes during helminth infection and identifies this subset as Tfh cells. Previously, it had been difficult to identify and localize IL-4–producing cells in situ. This is largely because of the fact that cytokines are rapidly released from the cells in which they are produced. Moreover, because of the almost ubiquitous expression of the IL-4 receptor (24), there is the inherent risk that cells that have bound IL-4 are incorrectly identified as IL-4 producers rather than responders. Like any experimental system, data obtained with cytokine reporter mice have to be interpreted carefully. It is, for example, possible that our inability to detect huCD2$^+$ cells in the T cell zones of mesLNs after infection results from a delay between the initiation of IL-4 production and huCD2 surface expression. However, this seems unlikely given our previous demonstration that robust huCD2 expression can be induced in vivo within minutes of stimulation (20). Conversely, huCD2 surface expression is maintained after the withdrawal of stimulation with an estimated half-life of 24 h (20). Collectively, it seems likely that local signals in the B cell areas induce huCD2 expression and suggests that Tfh cells are retained within the B cell follicle for extended periods of time.

In contrast to IL-4–producing cells, IL-4–competent T cells expressing GFP were widespread throughout the lymph nodes (Fig. 1). As we have previously shown, this subset can

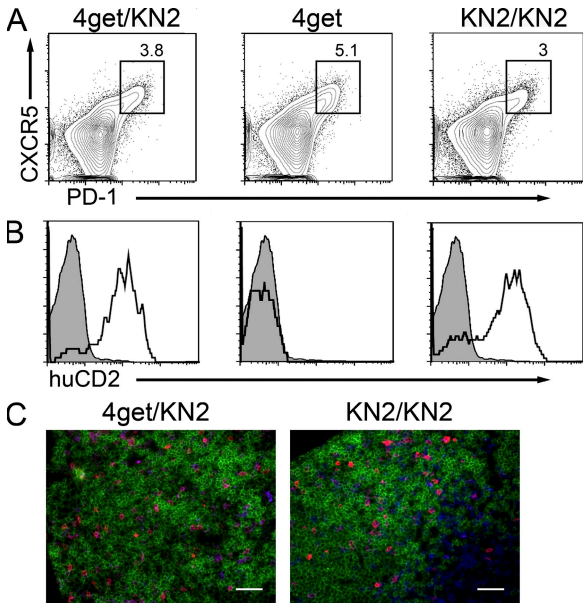

Figure 4. IL-4 is dispensable for Tfh cell generation. (A) mesLNs from 4get/KN2, 4get, and KN2/KN2 (IL-4–deficient) mice were harvested 14 d after *H. polygyrus* infection, and cells were stained for CXCR5 and PD-1 expression. Data shown are gated on CD4$^+$ cells. Numbers represent the frequency of the boxed population within the CD4$^+$ population. (B) huCD2 expression on CXCR5$^+$PD-1$^+$ cells (open histograms) as shown in A. CXCR5$^-$PD-1$^-$ cells (shaded histograms) are shown for comparison. (C) 4get/KN2 or KN2/KN2 mesLNs from *H. polygyrus*–infected animals stained with B220 (green), CD4 (blue), and huCD2 (red). Results shown are representative of two separate experiments with four mice per group. Bars, 50 μm.

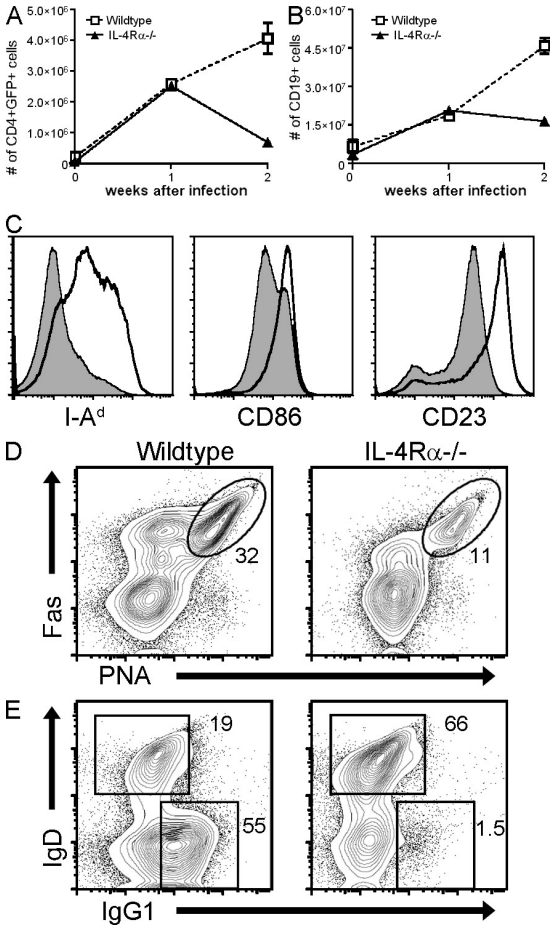

Figure 5. B cell expansion and maturation critically depend on IL-4 signaling. mesLNs from 4get/KN2 or IL-4Rα$^{-/-}$mice were harvested from naive animals 1 or 2 wk after *H. polygyrus* infection, and the total number of CD4$^+$GFP$^+$ (A) cells and CD19$^+$ (B) cells was calculated. Error bars represent the mean ± SEM. (C) mesLN CD19$^+$ cells stained for the indicated markers at 2 wk after infection. Open histograms represent cells from *H. polygyrus*–infected 4get/KN2 mice. IL-4Rα$^{-/-}$ B cells (shaded) are shown for comparison. (D and E) mesLN CD19$^+$ cells from *H. polygyrus*–infected 4get/KN2 and IL-4Rα$^{-/-}$ mice stained for PNA and Fas (D) or IgD and IgG1 (E). Numbers represent the frequency of the boxed population within the CD19$^+$ population. All plots shown are representative of at least three mice per group from two or more individual experiments.

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rapidly induce huCD2 expression upon stimulation (20). Furthermore, a high frequency of GFP$^+$huCD2$^+$ Th2 cells are present in effector sites after *H. polygyrus* infection, such as the lamina propria of the small intestine (20). Interestingly, Zaretsky et al. show that huCD2$^+$ cells in nonlymphoid effector sites do not display characteristics of Tfh cells (see Zaretsky et al. [26] on p. ■■■ of this issue). They also demonstrate the ability of IL-4–competent T cells to differentiate into Tfh cells in a germinal center–dependent manner after transfer into mice immunized with schistosome egg antigen (26). Collectively, these observations suggest that IL-4–competent cells within the T cell zone represent a pluripotent lineage of Th2 cells that must cross a critical checkpoint before progressing to an effector subset either within the lymph nodes or peripheral tissue. Whether this process is regulated by reencounter with antigen and/or alternative signals remains to be determined (23, 27).

In addition to the investigation into the localization of IL-4 production within the reactive lymph nodes, our report challenges the notion recently put forth by Nurieva et al. that Tfh cells develop independently from Th1, Th2, or Th17 cells (28). Indeed, it is clear from our and other studies that IL-4 is dispensable for Tfh generation (Fig. 4) (28). However, our data also indicate that IL-4 is important for maintaining the expansion and/or accumulation of Th2 and B cells over the course of a chronic infection. Nevertheless, in the context of an infection that elicits robust Th2 responses, we show that Tfh cells are the dominant if not exclusive IL-4–producing cells in the reactive lymph nodes and that IL-4 serves as a critical effector cytokine for a mature B cell response. Given the number of experimental settings in which Tfh cells have been shown to be generated, it stands to reason that this lineage of effector cells may withhold the ability to alter its effector phenotype to meet the demands of an appropriate host response (22, 28, 29). Future studies will undoubtedly continue to shed light on the relationship between Tfh cell differentiation and other T helper lineages.

The previously unappreciated compartmentalization of Th2 cell differentiation versus IL-4 production in the reactive lymph nodes represents an important advancement in our understanding of the two-step process for cytokine production we have previously described (20). The extent and timing of the B cell defects observed in IL-4Rα–deficient mice during the second week of infection illustrate how efficiently IL-4–producing Tfh cells populate the B cell follicles throughout the lymph nodes and govern the local environment. The colocalization of IL-4–producing Tfh and B cells is likely to optimize an integrated type 2 response by linking, for example, the IgE isotype switch to the broad up-regulation of CD23, the low affinity IgE receptor, on all B cells. These data in combination with the data from Zaretsky et al. (26) reveal an intricate interdependence between B and T cells in type 2 immunity, with IL-4 as a critical link. Of note, while our manuscript was under consideration, a study by Reinhardt et al. came to similar conclusions using the same IL-4 reporter system in different Th2-polarizing infection models (30). It will be important to determine which signals are provided by B cells in this mutual interaction.

MATERIALS AND METHODS

Animals and infection. 4get (5), KN2 (20), 4get/KN2, 4get × IL-4Rα$^{-/-}$ (25), and BALB/c mice were bred and kept under specific pathogen-free conditions at the Trudeau Institute, and were used at 8–12 wk of age. Animals were infected by gavage with 200 third-stage larvae of *H. polygyrus*, as previously described (20). All experiments were performed under Trudeau Institute Institutional Animal Care and Use Committee–approved protocols.

Flow cytometry. Single-cell suspensions were prepared from the mesLNs, stained and analyzed as previously described (20). The following mAbs were used for flow cytometry: CD4-PerCP, CD19-allophycocyanin (APC), hCD2-PE, CXCR5-biotin, Fas-PE, CD23-PE, CD86-PE, and streptavidin-PerCP (BD); I-A^b–biotin and streptavidin-APC (Invitrogen); lectin PNA–biotin (Vector Laboratories); PD-1–PE–Cy7 (BioLegend); IgD–biotin, ICOS-PE, and BTLA-PE (eBioscience); and IgG1-PE (SouthernBiotech). Samples were acquired on a FACSCanto II or FACSCalibur (BD) and were analyzed with FlowJo software (Tree Star, Inc.).

RT-PCR. cDNA was prepared as previously described (20). TaqMan reagents for *GAPDH* and IL-4 have been previously described (20), and BCL-6 and IL-21 primers and probes were Assays on Demand purchased from Applied Biosystems. Quantitative real-time RT-PCR was performed by using a TaqMan 7500 Fast System and software (Applied Biosystems). Fold expression was calculated using the $\Delta\Delta^{CT}$ method and *GAPDH* as a reference gene.

Immunohistochemistry. mesLNs were harvested from *H. polygyrus*–infected animals and immediately frozen in optimal cutting temperature (OCT) embedding compound (Sakura Finetek) over liquid nitrogen for detection of surface antigens. To detect intracellular GFP, whole lymph nodes from 4get mice were fixed in a solution of 4% formalin and 10% sucrose for 8 h before freezing. Frozen lymph nodes were cut into 5–8-mm sections on a cryostat (Leica) and fixed in a mixture of ice-cold 75% acetone/25% ethanol for 5 min. Sections were blocked in PBS plus 2% BSA and 5% 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 488 (Invitrogen), biotin mouse anti–human CD2 (clone RPA-2.10; BioLegend), and CD4-APC (clone RM4-5; eBioscience) in blocking buffer, followed by streptavidin–Alexa Fluor 568 (Invitrogen). All images were captured using a microscope (Axiovert 200M) and analyzed with Axiovision software (both from Carl Zeiss, Inc.), and represent data from three independent experiments.

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