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B Cell Antigen Presentation in the Initiation of Follicular Helper T Cell and Germinal Center Differentiation

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High-affinity class-switched Abs and memory B cells are products of the germinal center (GC). The CD4⁺ T cell help required for the development and maintenance of the GC is delivered by follicular Th cells (TFH), a CD4⁺ Th cell subset characterized by expression of Bcl-6 and secretion of IL-21. The cellular interactions that mediate differentiation of TFH and GC B cells remain an important area of investigation. We previously showed that MHC class II (MHCII)–dependent dendritic cell Ag presentation is sufficient for the differentiation of a TFH intermediate (termed pre-TFH), characterized by Bcl-6 expression but lacking IL-21 secretion. In this article, we examine the contributions of MHCII Ag presentation by B cells to TFH differentiation and GC responses in several contexts. B cells alone do not efficiently prime naive CD4⁺ T cells or induce TFH after protein immunization; however, during lymphocytic choriomeningitis virus infection, B cells induce TFH differentiation despite the lack of effector CD4⁺ T cell generation. Still, MHCII⁺ dendritic cells and B cells cooperate for optimal TFH and GC B cell differentiation in response to both model Ags and viral infection. This study highlights the roles for B cells in both CD4⁺ T cell priming and TFH differentiation, and demonstrates that different APC subsets work in tandem to mediate the GC response. The Journal of Immunology, 2014, 192: 000–000.

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and GC B cell differentiation and function (31, 38–41). The notion of unique B cell signaling has been challenged by other groups (42, 43), who instead suggest that T<sub>FH</sub> differentiation simply requires persistent TCR signals. Concretely delineating the requirement for individual MHCII<sup>+</sup> APCs to initiate and maintain T<sub>FH</sub> differentiation and development of the GC should resolve these conflicts.

In this study, we describe a novel mouse model in which MHCII, I-A<sup>B</sup>, is restricted to B cells. We define the ability of B cells to prime naive CD<sup>4</sup><sup>+</sup> T cells in vivo and the contribution of B cells to T<sub>FH</sub> differentiation in different contexts. MHCII expression restricted to B cells cannot drive CD<sup>4</sup><sup>+</sup> T cell priming, T<sub>FH</sub> differentiation, or initiate GC responses in response to nominal peptide and protein immunization. However, in the context of viral infection, B cell MHCII expression is sufficient to induce limited T cell priming and strikingly endows the vast majority of Ag-specific CD<sup>4</sup><sup>+</sup> T cells with a T<sub>FH</sub> phenotype. However, the generation of functional Ag-specific GCs and subsequent plasma and memory B cell output requires both DC and B cell MHCII expression. Therefore, in the setting of viral infection, MHCII<sup>+</sup> B cells may be able to drive the T<sub>FH</sub> program; however, MHCII-dependent Ag presentation by both DCs and B cells is necessary to induce optimal differentiation of T<sub>FH</sub> and GCs.

Materials and Methods

Mice

C57BL/6J, CD19 Cre, and OT-II mice were purchased from Jackson Laboratories. Smarta TCR transgenic mice (44), Foxp3 GFP mice (45), and CD11c/C<sup>A<sub>b</sub></sup> mice (6) were bred in-house. MHCII<sup>+</sup>C<sup>A<sub>b</sub></sup> STOP/STOP mice were developed at Washington University in St. Louis as described previously and subsequently bred in-house (46). B-MHCII mice were bred as CD19<sup>Cre</sup>/+; B/DC-MHCII mice additionally had the CD11c/C<sup>A<sub>b</sub></sup> transgene. Wild type (WT) control mice were bred as MHCII STOP/STOP mice were used at 8–18 wk of age.

Immunizations and infections

CD<sup>4</sup><sup>+</sup> OT-II T cells, CD<sup>4</sup><sup>+</sup> Smarta T cells, and CD<sup>4</sup><sup>+</sup> polyclonal cells from C57/BL6J mice were enriched by negatively selecting out CD<sup>8</sup><sup>+</sup>, B220<sup>+</sup>, MHCII<sup>+</sup>, and FcγRIII<sup>+</sup> cells, and labeled with CFSE where indicated, as previously described (48). OT-II cells were transferred i.v. 1 d before i.p. immunization with 50 μg NP<sub>E</sub>-OVA (4-hydroxy-3-nitrophenyl [acetyl coupled to OVA]; Biosearch Technologies) in alum (Sigma) as previously described (32, 36). A total of 1 × 10<sup>5</sup> Smarta cells was transferred i.v. 1 d before infection with 2 × 10<sup>6</sup> PFU lymphocytic choriomeningitis virus (LCMV) Armstrong (experiments shown in Fig. 4) or 2 × 10<sup>6</sup> PFU Armstrong (experiments shown in Fig. 6) as previously described (49). Virus was grown and titered as described previously (49). B-MHCII and B/DC-MHCII mice infected with LCMV Armstrong also received ∼10<sup>5</sup> CD<sup>4</sup><sup>+</sup> polyclonal T cells isolated from C57/BL6 mice 7–14 d before infection to reconstitute the CD<sup>4</sup><sup>+</sup> T cell compartment.

In vitro cultures

Sorted B cells were incubated overnight with 10 μg LPS and 1 d later were incubated with CFSE-labeled purified OT-II cells (see earlier Immunizations and Infections section), at a ratio of 1:10, with 10,000 B cells and 100,000 OT-II cells/well; OVA protein at a concentration of 100 μg/ml. CFSE dilution of OT-II cells was analyzed 4 or 5 d later.

Flow cytometry and cell sorting

All Abs were purchased from Biolegend, eBioscience, BD Pharmingen, or Invitrogen. DAPI or Live/Dead AQUA (Invitrogen) was used to identify live cells. The Fosp3<sup>+</sup> fixation and permeabilization kit was used to detect intracellular Bcl6 and Fosp3 staining (eBioscience). Cells were acquired or sorted on an LSR II cytometer or FACSaria II, respectively (BD Biosciences). Data were analyzed using FlowJo software (TreeStar). All FACS plots shown were gated on live, singlet cells.

Quantitative PCR

Quantitative PCR (qPCR) was conducted as previously described (36). In brief, RNA was extracted using the RNeasy Mini kit (Qiagen), and cDNA was made using the high-capacity cDNA reverse transcription kit (Applied Biosystems). GAPDH was used as the housekeeping gene for T<sub>FH</sub> cell qPCR, 18s was used as a housekeeping gene for all other qPCRs. qPCRs

![Figure 1](http://www.jimmunol.org/)
were performed on an 7500 Real Time PCR system machine (Applied Biosystems). Data were analyzed using the ΔΔ cycle threshold method.

**ELISPOT and ELISA assays**

For NP-specific ELISPOTS, splenocytes were incubated on 10 μg/ml NP25-BSA (high affinity) or NP25-BSA (all affinities; Biosearch Technologies)–coated plates (Millipore) and incubated with biotin anti-mouse IgG1 or IgM (Southern Biotech) followed by incubation with ExtrAvidin-Alkaline Phosphatase (Sigma) and developed with NBT/BCIP substrate (Sigma). Spots were enumerated on CTL-ImmuSpot reader (Cellular Technologies). LCMV-specific Abs were detected in serum by ELISA. Lysate from baby hamster kidney cells infected with LCMV Armstrong was used to coat ELISA plates. HRP-linked Abs against mouse IgG were used to detect the LCMV-reactive Abs. Relative OD values were determined at 450 nm, and values at dilutions within a linear range were used to determine final relative absorption.

**Results**

**Restricting MHCII expression to B cells**

To better study the requirements for various MHCII+ APCs in CD4+ T cell activation, we recently developed a new mouse strain Cre, MHCII Ab mediated recombination and cassette deletion. In the absence of the control of its own promoter and regulatory elements after Cre-p.i. Gated on CD19+B220+ cells (top plots) and further on GL-7+ IgD+ SEM.

106 OT-II cells was transferred to WT and B MHCII, and mice were immunized i.p. with 50 μg NP-OVA in alum. (A) Representative FACS plots of CFSE dilution of OT-II cells in the spleen on day 7 p.i. Gated on CD19+ TCRb+ CD90.1+ OT-II cells. (B) Representative FACS plots of OT-II cells to identify CXCR5+PD-1hi Tfh on day 7 p.i. Numbers indicated percentage of OT-II cells that are CXCR5+ PD-1hi. (C) Total number of OT-II Tfh in WT and B-MHCII mice on day 7 p.i. (D) Representative FACS plots of GL−7+GC B cells (top plots) and IgG1 expression and NP-specific cells of the GC (bottom plots) on day 7 p.i. Gated on CD19+B220+ cells (top plots) and further on GL−7+IgD+. Numbers represent the percentage of B cells that are GCs (top plots) and percent of GC B cells that are NP+ IgG1+ (bottom plots). (E) Total number of splenic NP-specific, IgG1+ GC B cells on day 7 p.i. (F) Total number of high-affinity, NP-specific IgG1+ ASCs per spleen. (G) Total number of IgM+ ASCs per spleen on day 14 p.i. as determined by ELISPOT. Bar graphs in (C) and (E)–(G) show mean ± SEM. n = 5–6 mice, data are pooled from two independent experiments. *p < 0.05, **p < 0.01, calculated using Student t test. ASC, Ab-secreting cell.
addition, the follicular and marginal zone B cell compartments in the spleen are also comparable between B-MHCII and WT mice (46).

To verify the functionality of B cells targeted with a “gene-repair cassette,” we examined the ability of B cells from B-MHCII mice to prime naive CD4+ T cells in vitro. CD19+ B220+ B cells were sorted from the spleens of WT and B-MHCII mice, activated overnight with LPS, pulsed with OVA protein, and incubated with CFSE-labeled, OVA-specific, TCR transgenic OT-II cells for 4 d. B cells from B-MHCII and WT mice proliferated to a similar extent after activation with LPS (data not shown). OVA-pulsed B cells from WT and B-MHCII mice induced a similar degree of OT-II proliferation (Supplemental Fig. 1D). Similar results were obtained using OVA peptide in place of OVA protein (data not shown). These data indicate that activated B cells from B-MHCII mice are functional and have the ability to process and present Ag to activate naive CD4+ T cells. In addition, WT and B-MHCII B cells induce comparable CD4+ T cell proliferation in vitro despite expressing different levels of MHCII.

B cells prime naive CD4 T cells poorly in response to nominal protein Ag in vivo

Although B cells are the most numerous MHCII+ APC in secondary lymphoid tissues, their contribution to the priming of naive CD4+ T cells in vivo remains unclear. The B-MHCII mice provide the ideal system to examine this question. Thymic cortical epithelium is MHCII+ in B-MHCII, which therefore lack a mature peripheral CD4+ T cell compartment (46). Despite the lack of conventional CD4+ T cells, B-MHCII mice have an intact CD8+ T cell compartment and normal lymphoid architecture, with segregation of T and B cells, as well as normal T cell zone and B cell follicle structure (data not shown), consistent with published data on mice lacking CD4+ T cells (36, 52). Given the lack of conventional CD4+ T cells in this system, we examined the response of adoptively transferred Ag-specific TCR transgenic CD4+ T cells. CFSE-labeled OT-II cells were transferred into MHCII-deficient, B-MHCII, or WT recipients 1 d before s.c. immunization with OVA protein emulsified in CFA. Four days p.i., OT-II cells in the draining LNs of WT mice had undergone extensive proliferation and expansion, whereas there was neither proliferation nor expansion of OT-II cells in the draining LNs of either B-MHCII mice or MHCII KO mice (Fig. 1A, 1B). Consistent with these data, OT-II cells in B-MHCII mice had significantly less CD44 expression than those found in WT mice, verifying defective activation (Fig. 1C).

Using a protein immunization system limits Ag delivery to the small number of B cells with a BCR specific for the immunizing Ag (53), and non-BCR–mediated Ag uptake mechanisms such as pinocytosis (54), which are quite inefficient. To examine T cell priming in a scenario in which all B cells could present peptide–MHCII complexes regardless of BCR specificity, we immunized mice i.v. with OVA 323–339 peptides and LPS. OT-II cells in WT mice exhibited extensive proliferation, with most of the cells found in the fourth division or greater (Fig. 1D). In contrast, OT-II cell proliferation induced by B cells alone in B-MHCII mice was suboptimal because the majority of cells had divided only once or twice (Fig. 1D). OT-II cells primed by B cells did have increased CD44 expression in comparison with mice that were not immunized, although they expressed much less CD44 than OT-II cells primed in WT mice (Fig. 1F) and produced significant IFN-γ and IL-2 (Supplemental Fig. 3). However, there was no increase in the number of OT-II cells in either the spleen or peripheral LNs (Fig. 1E) of immunized B-MHCII mice compared with unimmunized mice. Thus, B cells are capable of inducing minimal CD4+ T cell priming in vivo when directly targeted with processed Ag, but B cell Ag presentation alone does not induce the activation and expansion observed when other MHCII+ APC populations are also functional.

B cell–restricted Ag presentation is not sufficient to elicit TFH and GC formation after peptide or protein immunization

We considered the possibility that B cells could interact with T cells to induce TFH and GC differentiation, despite their inability to generate significant CD4+ T cell expansion. To address this, we examined the response of OT-II cells and Ag-specific B cells p.i. with haptenated NP-OVA in alum, which elicits strong GC and Ab responses. Differentiation of OT-II TFH and GC B cells was examined 7 and 14 d p.i. Similar to i.v. immunizations, OT-II cells in B-MHCII mice underwent minimal proliferation and no expansion after NP-OVA immunization (Fig. 2A, 2C). In addition, upregulation of CXCR5 was impaired on OT-II cells primed in B-MHCII mice, and there was no differentiation of CXCR5+ PD-1+ TFH on either day 7 or day 14 p.i. (data not shown). In the absence of TFH, neither Ag-specific GCs (Fig. 2D, 2F) nor high-affinity PCs in the

![FIGURE 3. Minimal T<sub>FH</sub> differentiation in response to peptide immunization. A total of 2 × 10<sup>6</sup> CFSE-labeled OT-II cells was transferred to WT and B-MHCII mice. Mice were immunized i.v. with 100 µg OVA 323–339 peptide and 75 µg LPS. Splenocytes were examined 7 d p.i. (A) Representative FACS plots of CD19<sup>+</sup> TCRβ<sup>+</sup> CD4+ OT-II cells to identify CXCR5<sup>+</sup> PD-1<sup>+</sup> T<sub>FH</sub> cells. CXCR5<sup>+</sup> and CXCR5<sup>+</sup> OT-II cells were sorted from WT and B-MHCII mice p.i. and examined for (B) Bcl6 mRNA and (C) IL-21 mRNA. Bar graphs in (A) and (B) show mean ± SEM. n = 3–4 mice/group, representative of three independent experiments.](http://www.jimmunol.org/)
B cell–restricted Ag presentation induces TFH differentiation post-viral infection

Immunization with model Ags in adjuvant is a useful tool for understanding the biology of an immune response, but it does not always mimic the processes that occur in the context of infection. To examine B cell–restricted Ag presentation during acute viral infection, we reconstituted the CD4+ T cell compartment of B-MHCII mice with 10^7 polyclonal CD4+ T cells and transferred 1 × 10^6 LCMV GP61–80 specific Smarta TCR Tg T cells to WT and B-MHCII mice 1 d before infection with LCMV Armstrong. On day 8 postinfection, there was much less expansion of Smarta T cells in infected B-MHCII mice than in WT littersmates with ~100 times fewer cells (Fig. 4C). However, Smarta cells did not expand in infected MHCII KO mice; thus, the expansion observed in B-MHCII mice was Ag specific. Strikingly, upward of 90% of the Smarta cells in B-MHCII spleens exhibited a TFH phenotype (Fig. 4A, 4C). TFH cells primed in WT and B-MHCII mice had equivalent levels of Bcl-6 mRNA (data not shown) and protein (Fig. 4D), and also expressed equivalent levels of IL-21 mRNA (Fig. 4E), suggesting that the CXCR5+ cells primed by B cells were indeed TFH cells. Overall, these data demonstrate that in the setting of acute viral infection, B cells can induce partial TFH differentiation and skew T cells almost exclusively toward the TFH lineage.

Because TFH play a critical role in the GC B cell response, we next asked whether LCMV-specific GC responses were present in LCMV-infected B-MHCII mice. Because there are no reagents to assay LCMV-specific B cells by FACS, we quantified the number of GL-7+ IgDlo B cells in spleens of WT and B/DC-MHCII mice by FACS and measured serum IgG Abs by ELISA on day 8 post-infection. Postinfection, WT mice generated significant numbers of IgDlo GL-7+ GC B cells; however, B-MHCII mice had almost no GC B cells, close to the background level observed in uninfected mice (Fig. 4F). Consistent with these data, B-MHCII mice generated only minimal LCMV-specific IgG Abs titers, although greater than the levels in uninfected mice (Fig. 4G). Thus, the small number of TFH cells generated in B-MHCII mice after LCMV infection was insufficient for GC formation.

The combination of DC and B cell Ag presentation is sufficient for Tfh differentiation and GC development after protein immunization

Previous work has shown that generation of a partially differentiated Tfh cell (pre-Tfh) (36) is initiated by MHCII+ DCs before cognate T–B interactions (31, 34–36). We and others have proposed that B cell Ag presentation completes the Tfh program (21, 36). However, the ability of MHCII+ B cells to complete Tfh differentiation has not been directly examined. We therefore crossed B-MHCII mice to mice in which only CD11c^hi lymphoid-resident DCs are MHCII+ (DC-MHCII, referred to as CD11c^hi A^hi) (6, 36), to generate mice in which MHCII is expressed by conventional DCs and B cells together (B/DC-MHCII mice). To examine Tfh differentiation in the presence of DC and B cell MHCII expression, we again analyzed transferred OT-II

**FIGURE 4.** B cell Ag presentation preferentially drives Tfh differentiation in response to viral infection. B-MHCII and MHCII KO mice received 1 × 10^7 CD4+ T cells from C57/BL6 mice 7–14 d before infection to reconstitute the CD4+ T cell compartment. A total of 1 × 10^6 SMARTA transgenic CD4+ T cells was transferred to WT and B-MHCII mice, and the mice were infected with LCMV Armstrong 1 d later. Splenocytes were analyzed on day 8 postinfection. (A) Representative FACS plots of CD19^- TCRB^- CD4+ SMARTA cells to identify CXCR5+ PD-1+ Tfh cells. (B) Percentage of SMARTA cells in WT and B-MHCII mice that are CXCR5+ PD-1+ Tfh cells. (C) Total number of splenic SMARTA Tfh cells in WT, B-MHCII, and MHCII KO mice. (D) Histogram overlay of Bcl6 expression by CXCR5+ PD-1+ SMARTA Tfh cells. (E) Relative expression of IL-21 mRNA in sorted CXCR5+ PD-1+ SMARTA cells. (F) Total number of CD19^+ B220^+ IgDlo GL-7+ GC B cells. (G) Measurement of LCMV-specific IgG in the serum on day 8 p.i., compared with uninfected C57/BL6 mice. Bar graphs show mean ± SEM. Data are representative of two independent experiments with three to six mice per group.

*p < 0.05, **p < 0.01, ***p < 0.001, calculated using Student t test.
cells in mice immunized i.p. with NP-OVA in alum. OT-II cells expanded similarly in DC-MHCII, B/DC-MHCII, and WT mice (Fig. 5A) and generated similar numbers of CXCR5+ OT-II cells with equivalent expression of Bcl6 mRNA and protein (Fig. 5B, 5C, 5E, 5F). Consistent with our prior work, Ag-specific CD4+ T cells primed by DCs alone lack the PD-1hi TFH population found in WT mice (Fig. 5B, 5D); however, PD-1hi TFH are restored in B/DC-MHCII mice (Fig. 5B, 5D). Although CXCR5+ OT-II cells primed only by DCs exhibit approximately a 10-fold reduction in IL-21 mRNA levels when compared with WT-TFH, TFH primed by both DCs and B cells exhibit similar levels of IL-21 transcript as WT-TFH (Fig. 5G). Together, these data demonstrate that MHCII+ DCs and B cells cooperate for TFH differentiation p.i., because neither population alone is sufficient for TFH differentiation, but the combination is.

Because TFH function to drive and sustain the GC B cell response, we hypothesized that the combination of DC and B cell Ag would also suffice for differentiation of GC B cells. Indeed, 7 d p.i., both WT and B/DC-MHCII spleens contained equivalent numbers of Fas+ IgDlo NP-binding, IgG1+ GC B cells (Fig. 6A, 6B). GCs function to generate high-affinity class-switched PCs and memory B cells. Fourteen days after NP-OVA immunization, there were similar numbers of high-affinity IgG1+ NP+ Ab-secreting cells in the spleen (Fig. 6C), as well as in the BM (data not shown) of WT and B/DC-MHCII mice. Similarly, on day 14 p.i. (data not shown), as well as day 29 p.i. (Fig. 6D, 6E), B/DC-MHCII spleens contained NP-binding IgG1+ memory B cells in similar numbers to WT mice. In combination with our published data, these data suggest that MHCII expression by both DCs and B cells are both necessary and sufficient for GC B cell differentiation after protein immunization.

**DC and B cell Ag presentation during viral infection**

Because B cell priming alone was insufficient to induce optimal TFH or Ab responses after acute LCMV infection, we hypothesized that the addition of DC Ag presentation was necessary. We therefore compared WT and B/DC-MHCII mice acutely infected with 2 × 10^4 PFU LCMV Armstrong. Smarta cells had expanded equivalently in WT and B/DC-MHCII mice on day 8 postinfection (Fig. 7A), and similar numbers of Smarta cells had differentiated into CXCR5+ PD-1hi Bcl6+TFH cells in B/DC-MHCII and WT mice (Fig. 7B–D), indicating that DC and B cell MHCII expression is sufficient for TFH differentiation in the setting of viral infection.

Because DC and B cell MHCII expression was sufficient for Ag-specific GC B cell responses to immunization, we asked whether this was also true post-viral infection. Although GC B cells did develop in B/DC-MHCII mice, the GC population was significantly smaller than in WT mice (Fig. 7E). In agreement, B/DC-MHCII mice generated lower titers of IgG+ LCMV-specific Abs than did WT mice.

**FIGURE 5.** MHCII Ag presentation by DCs and B cells cooperates for TFH differentiation. A total of 1 × 10^5 OT-II cells was transferred to WT, B-MHCII, DC-MHCII, and B/DC-MHCII mice. Mice were immunized with NP-OVA in alum i.p. and analyzed on day 7 p.i. (A) Total number of OT-II cells (CD19− TCRβ+CD90.1+) in the spleen on day 7 p.i. (B) Representative FACS plots of OT-II cells for expression of CXCR5 and PD-1 to identify TFH. Numbers represent the percent of OT-II cells that are CXCR5+ PD-1hi and PD-1int. (C) Total number of CD62L− CXCR5+ OT-II cells in the spleen on day 7 p.i. (D) Quantification of PD-1hi OT-II TFH from the plots shown in (B). (E) Histogram overlay of Bcl6 expression by CD62L− CXCR5+ OT-II cells. Relative expression of (F) Bcl6 and (G) IL-21 mRNA in sorted CXCR5+ OT-II cells relative to naive CD4+ T cells. Bar graphs in (A), (C), (D), (F), and (G) show mean ± SEM. n = 3–5 mice/group, representative of three to four independent experiments. ***p < 0.001, calculated using a one-way ANOVA with Tukey’s analysis.
WT mice, although the levels were significantly greater than those of uninfected mice (Fig. 7F). We suspect the decreased GC responses in B/DC-MHCII mice represent the limitations of reconstituting the T cell compartment with transferred CD4+ T cells and the requirement for viral-specific CD4+ T cells of multiple different specificities with diverse Ag-specific B cells in the GC response. Nonetheless, MHCII+ DCs and B cells do generate both T<sub>FH</sub> and GCs post-viral infection, in contrast with MHCII<sup>+</sup> B cells alone.

**B/DC-MHCII mice have increased GCs in the absence of peripheral regulatory T cells**

Follicular regulatory T cells (T<sub>FR</sub>) express Foxp3 and Bcl6, and localize to the GC to limit the humoral response mediated by T<sub>FH</sub> and GCs. However, B/DC-MHCII mice do not have these regulatory T cells. Therefore, the increased GC responses observed in these mice are likely due to the absence of regulatory T cells.
B cells. (D) specific IgG1+ GCs on day 14 p.i. as quantified from the plots in (B/DC-MHCII mice, and mice were immunized with NP-OVA in alum. (A) Numbers represent the percentage of B cells that are GCs (top plots) and percent of GC B cells that are NP+ IgG1+ (bottom plots). (B) WT CD4+ cells, (containing 10–15% Foxp3+ Tregs) (58), which resulted in normalization of the numbers of both OT-II T cells and CD4+ T cells and Tregs. In parallel, B/DC-MHCII mice also had increased numbers of OT-II T cells; the numbers of OT-II T cells were also increased in B/DC-MHCII mice, but this reflected the overall increase in OT-II cells rather than a selective increase in T FR (Fig. 8D). Although B/DC-MHCII and WT spleens contained a similar number of Ag-specific NP+ GC B cells, B/DC-MHCII mice also had a large number of NP+ IgG1+ GC B cells (Fig. 8A). Thus, the ratio of NP-binding to NP-negative cells within the IgG1+ GC population was significantly reduced in B/DC-MHCII mice (Fig. 8C), indicating an outgrowth of NP nonbinding clones in the absence of endogenous CD4+ T cells and Tregs.

We reconstituted B/DC-MHCII mice with 1×10^7 polyclonal WT CD4+ cells, (containing ~10–15% Foxp3+ Tregs) (58), which resulted in normalization of the numbers of both OT-II T cells and GC B cells (Fig. 9A, 9B, 9D). The ratio of NP+ to NP- GC B cells also returned to WT levels (Fig. 9C). We hypothesized that the presence of T FR in the polyclonal CD4+ T cells transferred into B/DC-MHCII mice was responsible for the normalization of the GC response. To directly determine whether Foxp3+ T cells could mediate this process, we transferred 5×10^6 Foxp3+ GFP+ Tregs from WT Foxp3+ GFP reporter mice (45) (a number equivalent to ~5×10^6 bulk CD4+ T cells) in addition to 1×10^7 OT-II cells and immunized the mice with NP-OVA. On day 14 p.i., GC numbers in B/DC-MCHIII mice were reduced to the levels of WT in those mice that also received Foxp3+ Tregs (data not shown), although this difference was more variable than B/DC-MHCII mice that received polyclonal CD4+ T cells. However, the transfer of Foxp3+ Tregs increased the ratio of NP+ to NP- IgG1+ GC B cells to approximately that of WT mice (Fig. 9E). These data confirm and support a critical role for T FR cells in the control of the GC response.

Overall, these data support previous observations that describe a role for regulatory T cells in the control of the GC response. They also agree with a previous observation that T FR cannot differentiate from activated OT-II cells but differentiate from previously generated Tregs (Fig. 9F) (55). These results also demonstrate that the MHCII-dependent interaction of T FR with DCs and/or B cells is sufficient for T FR to exert their function in the GC and that MHCII expression by other cell types is not required.

**Discussion**

In this study, we investigated the role for B cell Ag presentation in naive CD4+ T cell priming, T FR differentiation, and development

**FIGURE 8.** Increased OT-II and GC responses in the absence of endogenous CD4+ T cells. A total of 1×10^7 OT-II cells was transferred to WT and B/DC-MHCII mice, and mice were immunized with NP-OVA in alum. (A) Representative FACS plots of splenic GC B cells (gated on CD19+ B220+ splenocytes, top plots), IgG1 expression, and NP-specific cells of the GC population (gated on CD19+ B220+ Fas+ IgDlo cells, bottom plots) on day 14 p.i. Numbers represent the percentage of B cells that are GCs (top plots) and percent of GC B cells that are NP+ IgG1+ (bottom plots). (B) Total number of NP-specific IgG1+ GCs on day 14 p.i. as quantified from plots in (A). (C) Ratio of the percentage of NP+ to NP- cells of CD19+ B220+ Fas+ IgDlo GC B cells. (D) Representative FACS plots of CD19+ TCRβ+ OT-II cells (top plots) and CXCR5+ PD-1hi OT-II T cells (bottom plots) on day 14 p.i. Numbers represent the percent of CD4+ T cells that are OT-II (top plots) and the percent of OT-II cells that are CXCR5+ PD-1hi (bottom plots). (E) Total number of splenic CD19+ TCRβ+ OT-II cells on day 14 p.i. as quantified from plots in (D), Bar graphs in (B), (C), and (E) show mean ± SEM. n=5–6 mice/group, representative of two independent experiments. *p<0.05, **p<0.01, calculated with Student’s t-test.
of the GC. We found that MHCII Ag presentation restricted to B cells mediates very inefficient CD4+ T cell priming in response to either nominal protein or peptide Ags, without the induction of either T FH or a GC response. However, in response to acute viral infection, B cell Ag presentation skews the Ag-specific T cell response toward the T FH subset. Nevertheless, MHCII expression restricted to DCs and B cell mediates optimal T FH differentiation and expansion, as well as B cell GC formation with affinity maturation and isotype switching of Ag-specific B cells in response to immunization and viral infection. These studies highlight the requirement for cooperation among multiple cells during the initiation of a humoral immune response.

The ability of B cells to activate naive CD4+ T cells has been previously examined with conflicting results. It has been shown that B cells are poor CD4+ activators (19) and may tolerate CD4+ T cells (53, 59). However, others have demonstrated that LPS-activated B cells can activate CD4+ T cells in vitro (60), in agreement with our in vitro data. Teleologically, the inability of B cells to efficiently prime T cells is somewhat perplexing because they are the most numerous professional APC in secondary lymphoid tissues. The inability of B cells to prime naive CD4+ T cells p.i. may reflect the absence of an appropriate combination of costimulatory molecules and inflammatory cytokines expressed by DCs or may simply be a problem of anatomy because T and B cells are found in different locations in secondary lymphoid tissues. In response to acute viral infection, inflammation and the disruption of lymphoid architecture may enhance the activation of naive, Ag-specific B cells and permit them to interact with Ag-specific T cells (61). Thus, the reasons for the inability of B cells to effectively prime naive CD4+ T cells are not clear but may be a combination of location and signal quality.

Our data demonstrate that Ag presentation by DCs and B cells together is sufficient for optimal T FH differentiation in multiple settings, although the role of B cell Ag presentation in the process may be different p.i. and in response to infection. Recent studies have demonstrated that the differentiation of T FH precursors requires DCs and is initiated before interactions with B cells (31, 34, 35, 62). In agreement with these latter studies, we also identified a pre-T FH in mice with MHCII Ag presentation restricted to DCs (36). Multiple recent investigations have examined the requirement for B cells in the differentiation of T FH. Earlier studies had demonstrated that mice lacking B cells or the ability to maintain T-B conjugates lack T FH (24, 63). In addition, examination of gene-deficient mice also suggested that B cell expression of the costimulatory molecules, ICOS and PD-L2, was necessary for T FH differentiation (30, 31).

These data suggest that DCs and B cells may provide qualitatively distinct signals to T cells that contribute to T FH differentiation.

**FIGURE 9.** GCs and OT-II cell responses in the presence of either polyclonal CD4+ T cells or Tregs. (A-D) A total of 1 × 10^7 CD4+ T cells from WT mice was transferred to B/DC-MHCII mice. One week later, 1 × 10^6 OT-II cells were transferred to WT and B/DC-MHCII mice, and mice were immunized with NP-OVA/alum. Mice were analyzed on day 14 p.i. (A) Representative FACS plots of CD19+/B220+ splenic GC B cells (top plots) and NP-specific cells in the GC (bottom plots). Numbers represent the percentage of B cells that are GCs (top plots) and percent of GC B cells that are NP+ IgG1+ (bottom plots). (B) Total number of NP-specific IgG1+ GCs on day 14 p.i. quantified from the plots in (A). (C) Ratio of NP+ to NP– cells of CD19+ B220+ IgDlo Fas– IgG1+ GC B cells. (D) Representative FACS plots of OT-II cells (top plots) and OT-II T FH (bottom plots) on day 14 p.i. Numbers represent the percent of CD4+ T cells that are OT-II (top plots) and the percent of OT-II cells that are CXCR5– PD-1hi (bottom plots). (E) A total of 5 × 10^3 sorted GFP+ Foxp3+ Tregs from Foxp3 GFP mice and 10^5 OT-II cells was transferred to WT and B/DC-MHCII mice, and mice were immunized with NP-OVA in alum. Spleens were analyzed on day 14 p.i. Ratio of the percentage of NP+ to NP– cells of CD19+ B220+ IgDlo Fas– IgG1+ GC B cells. (F) Analysis of Foxp3 and CXCR5 of endogenous CD4+ T cells (gated on TCRβ+ CD19+ splenocytes) and OT-II cells (gated on TCRβ+ CD19– CD4+ CD090.1+) from the spleens of C57BL/6 mice immunized with NP-OVA in alum on day 8 p.i. Numbers represent the percentage of Foxp3+ CXCR5+ cells. Bar graphs in (B), (C), and (E) show mean ± SEM. n = 3–6 mice/group, representative of two experiments. Data in (E) are pooled from two independent experiments. *p < 0.05 using a one-way ANOVA with Tukey's analysis.
For example, IL-6, presumably produced by DCs, has an in vitro role in the induction of Bcl6 and may contribute to the differentiation of Tfh after protein immunization (64–66). However, more work has been done to identify costimulatory molecules expressed by B cells that may affect Tfh differentiation. B cells can provide many signals to Tfh, and one specific ligand–receptor pair may not be responsible. ICOS/ICOS ligand signals have been implicated in GC formation and IL-21 production (31, 38–40), and other receptor–ligand pairs, including PD-1 and its ligands, as well as CD80, are important in Tfh and GC B cell differentiation (21, 22, 30, 67). Although it has been suggested that Tfh differentiation does not require unique B cell signals but rather sustained Ag presentation (42, 43), most studies support the alternative model that cognate, Ag-specific B cells maintain Tfh that differentiate early after DC interactions. The striking observation described in this study that B cell–restricted Ag presentation exclusively primes Tfh cells, at the expense of Cxcr5+ effector T cells after viral infection, suggests that B cells may express and provide unique signals to T cells to induce the Tfh program. B cells alone exclusively generated Tfh cells postinfection but at greatly reduced numbers. The addition of DC Ag presentation is sufficient to induce optimal Ag-specific T cell expansion postinfection, as well as restore the normal proportion of Tfh and effector T cells. Therefore, DCs drive CD4+ effector T cell differentiation and T cell expansion postinfection, whereas B cell Ag presentation is the force behind Tfh differentiation. The Ag presentation requirements for GC B cell differentiation largely parallel those required for optimal Tfh differentiation. After protein immunization, the combination of DC and B cell MHCII expression is necessary and sufficient for the differentiation of functional GCs. However, despite the fact that B/MHCII mice were able to induce Ag-specific Tfh cells post-viral infection, they were unable to form GCs and LCMV-specific IgG Ab. This may be due to the fact that overall numbers of SMARTA Tfh were greatly reduced in B/MHCII mice compared with WT mice. The addition of DC Ag presentation was able to induce some GCs and Ag-specific IgG after LCMV infection, but this response was still less than that observed in WT mice. It is possible that other MHCII cells are required for GC formation during viral infection. However, we presume that this reflects an incomplete CD4+ T cell compartment in B/DC-MHCII mice. Given the demonstrated requirement for cognate B–T interactions in the GC, the bulk CD4+ T cells that we transferred probably contain insufficient numbers of CD4+ T cells specific for many LCMV epitopes. Thus, GC B cells and class-switched Abs are produced, but at reduced frequencies. These data do highlight the limitations of the protein immunization system and show that the minimal MHCII requirements for GC differentiation and functional Ab responses may be context dependent.

Finally, previous studies have demonstrated increased GC and T cell responses in the absence of Tfh (55–57), and our data also suggest a role for these cells. In the absence of endogenous CD4+ T cells, including naturally occurring Tregs, we observed increased Ag-specific T cells in the absence of Tregs, including an increase in Tfh cells, associated with increased GCs. The abundance of both GCs and T cells can be rectified by reconstituting a polyclonal CD4+ T cell population (which includes Foxp3+ Tregs) or by adding back only Foxp3+ Tregs. Because an abundance of Tfh cells is linked to autoantibody production (38), Tfh cells may play a critical role in the prevention of autoimmunity. The system we have developed will allow for further study of the role of Tfh cells in other contexts, as well as dissecting the role(s) of DC and B cell Ag presentation in other settings.

The results described in this article highlight the controlled and cooperative nature of CD4+ T cell activation, Tfh differentiation, and germinal B cell formation after protein immunization and LCMV infection. It remains to be seen, however, if these same requirements are also in place in the context of other infections, autoimmunity, or acute inflammation. One might imagine that in the setting of inflammation and disruption of the lymphoid tissue architecture, such as toxoplasma gondii (68), B cells may contribute to the activation of naive CD4+ T cells. In addition, the stringent requirements for Tfh activation may be altered in infection or autoimmunity, and perhaps a signal from either a DC or a B cell is sufficient for Tfh differentiation. The multiple steps required in Tfh differentiation may serve as a checkpoint in the prevention of autoimmunity by ensuring the Ag specificity of responding Tfh and ensuring that they make IL-21 only when it is appropriate.

Disclosures
The authors have no financial conflicts of interest.

References
Supplemental Figure 1: MHCII expression in B-MHCII mice (A) MHCII expression of splenic B cells (TCRβ-B220⁺) in WT, B MHCII and MHCII AβSTOP/STOP mice. Numbers indicate % of B cells that express MHCII (B) MHCII AβmRNA expression in sorted splenic B cells (CD19⁺B220⁺), normalized to WT cells. (C) MHCII AβmRNA expression in sorted splenic non-T, non-B cells (CD19⁻B220⁻TCRβ⁻), normalized to WT cells (D) Sorted splenic B cells from the indicated mice with activated with LPS and then pulsed with OVA protein and incubated with CFSE labeled OT-II cells. CFSE proliferation was assessed on d4. Bar graphs in (B) and (C) show mean ± SEM. Representative of 2 independent experiments (A,C,D) and <5 independent experiments (B), with 2-5 mice per group.
**Supplemental Figure 2**: MHCII expression of APC populations. APCs from WT, B-MHCII mice and MHCII AβbSTOP/STOP mice were examined for MHCII expression in the peripheral LNs, Peyer’s patches, mesenteric LNs, small intestine lamina propria and spleen. After gating on live singlets, the populations were identified as follows: Macrophages were identified as B220^{-} TCRβ^{-} CD11c^{+}, CD11b^{+}, F4/80^{+}, Gr-1^{-} DC were identified as B220^{-} TCRβ^{-} CD11c^{hi} and were further gated on CD4 and CD8 as noted. B cell were identified as B220^{+} TCRβ^{+}. Basophils were identified as B220^{+} TCRβ^{+} CD49b^{+} FCεR1^{+} cells. MHCII AβbSTOP/STOP mice are represented with a shaded gray histogram, B-MHCII mice by a blue histogram and WT mice by a red histogram. Representative of two independent experiments.
**Supplemental Figure 3:** Minimal cytokine production by T cells primed only by B cells. 2x10⁶ CFSE labeled OT-II cells were transferred to WT, B-MHCII and MHCII KO mice. Mice were immunized i.v. with 100μg OVA 323-339 peptide and 75μg LPS and splenocytes were analyzed on d7 p.i. (A) Interferon gamma production (IFNg) (top) and IL-2 production (bottom) by CD19⁺ TCRβ⁺ OT-II cells in the spleen 7 days after OVA peptide immunization after re-stimulation with PMA and ionomycin the presence of Brefeldin A. Percentage of OT-II cells primed in WT and B-MHCII mice that produce IFNg (B) and IL-2 (C) 7 days after immunization with OVA peptide in LPS. Bar graphs in (B) and (C) show mean ± SEM n= 4-5 mice per group, representative of 2-3 independent experiments. * indicates a p value of <0.05, ** indicates a p value of <0.01 calculated using Student’s t test.