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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 (T FH), a CD4+ Th cell subset characterized by expression of Bcl-6 and secretion of IL-21. The cellular interactions that mediate differentiation of T FH 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 T FH intermediate (termed pre-T FH), 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 T FH differentiation and GC responses in several contexts. B cells alone do not efficiently prime naive CD4+ T cells or induce T FH after protein immunization; however, during lymphocytic choriomeningitis virus infection, B cells induce T FH differentiation despite the lack of effector CD4+ T cell generation. Still, MHCII+ dendritic cells and B cells cooperate for optimal T FH 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 T FH differentiation, and demonstrates that different APC subsets work in tandem to mediate the GC response. The Journal of Immunology, 2014, 192: 3607–3617.

In addition to acting as effector cells, CD4+ T cells play a central role in immune responses by providing help to other cells, including B cells. Naive CD4+ T cells must be activated by APCs expressing peptide–MHC class II (MHCII) complexes. MHCII-dependent T cell–effector cell interactions are also required for the delivery of CD4+ T cell help. MHCII+ dendritic cells (DCs) are uniquely positioned to activate naive CD4+ T cells (1). However, multiple cell types express MHCII, including B cells, macrophages, basophils, mast cells, and some endothelial cells (2–4), and could mediate CD4+ T cell effector functions.

Multiple studies have shown that B cell expression of MHCII is necessary for B cells to “receive” CD4+ T cell help to mediate functions such as isotype class switching (5, 6). However, experiments to define the converse ability of MHCII+ B cells to present Ag to CD4+ T cells and drive T cell differentiation have yielded conflicting results (7). Early studies in mice lacking B cells suggested that B cells are required for optimal CD4+ T cell responses, including both initial priming and effector functions (8–16). Contrasting studies in B cell–deficient mice and allogeneic transfer systems in mice and chickens suggested that B cells activate T cells inefficiently and CD4+ T cell priming was independent of B cells (17–20). However, studies to directly test the sufficiency of B cell Ag presentation in CD4+ T cell priming are lacking.

Primed CD4+ T cells differentiate into multiple effector subsets, including follicular Th cells (T FH) (21, 22). T FH are necessary to initiate and maintain germinal centers (GCs), structures in secondary lymphoid tissues in which activated B cells undergo class switching and somatic hypermutation to generate high-affinity plasma cells (PCs) and memory B cells (23). T FH express the transcription factor Bc16, which controls their differentiation (24–26), the chemokine receptor CXCR5, allowing them to localize to the CXCL13-rich B cell follicles (27–29), as well as costimulatory molecules, including CD40L, ICOS, and programmed cell death 1 ligands (30–33). GC B cells are also required for the formation and function of the GC. Because T FH play a critical role in the GC process, it is important to understand the cells and cues that mediate their differentiation.

T FH differentiation is initiated early in the immune response, before CD4+ T cell interactions with B cells (31, 34, 35). Consistent with these observations, we previously showed that T FH differentiation requires DCs (36). However, DC priming is not sufficient to complete T FH differentiation, but instead drives the production of pre-T FH, a partially differentiated intermediate that expresses CXCR5 and Bc16 (36). Pre-T FH lack expression of PD-1 and do not produce significant quantities of cytokines, IL-21. It has been proposed that B cells mediate the differentiation of pre-T FH into IL-21–producing T FH. Several groups have demonstrated that Ag-specific B cells are necessary for T FH maintenance (24, 29, 31, 32, 37). Similarly, B cell expression of costimulatory molecules, including ICOSL, PD-1 ligands, and CD80, are necessary for T FH differentiation.
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 TFH differentiation simply requires persistent TCR signals. Concretely delineating the requirement for individual MHCII+ APCs to initiate and maintain TFH differentiation and development of the GC should resolve these conflicts.

In this study, we describe a novel mouse model in which MHCII, I-Aβ, is restricted to B cells. We define the ability of B cells to prime naive CD4+ T cells in vivo and the contribution of B cells to TFH differentiation in different contexts. MHCII expression restricted to B cells cannot drive CD4+ T cell priming, TFH 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 CD4+ T cells with a TFH 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+ B cells may be able to drive the TFH program; however, MHCII-dependent Ag presentation by both DCs and B cells is necessary to induce optimal differentiation of TFH and GCs.

Materials and Methods

Mice

C57BL/6J, CD19 Cre, and OT-II mice were purchased from Jackson Laboratories. Sm t a TCR transgenic mice (44), Foxp3 GFP mice (45), and CD11c/Aβ mice (6) were bred in-house. MHCII Aβ 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 CD11c/Aβ b STOP/STOP; B/MHCII mice additionally had the CD11c/Aβ b transgene. Wild type (WT) control mice were bred as MHCII STOP/STOP. MHCII Aβ b KO mice were (–), and MHCII Aβ b STOP as STOP. Mice were housed under pathogen-free conditions, in accordance with the University of Pennsylvania Animal Care and Use Guidelines, and were used at 8–18 wk of age.

Immunizations and infections

CD4+ OT-II T cells, CD4+ Smarta T cells, and CD4+ polyclonal cells from C57/BL6J mice were enriched by negatively selecting out CD8+, B220+, MHCII+, and FcγRII+ 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 NPb-OVA (4-hydroxy-3-nitrophenyl [acetyl coupled to OVA]; Biosearch Technologies) in alum (Sigma) as previously described (32, 36). A total of 1 × 106 Smarta cells was transferred i.v. 1 d before infection with 2 × 10⁵ PFU lymphocytic choriomeningitis virus (LCMV) Armstrong (experiments shown in Fig. 4) or 2 × 10⁵ 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 ~10⁶ CD4+ polyclonal T cells isolated from C57/BL6J mice 7–14 d before infection to reconstitute the CD4+ 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, in 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 Foxp3 fixation and permeabilization kit was used to detect intracellular Bcl6 and Foxp3 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 TFH cell qPCR, 18s was used as a housekeeping gene for all other qPCRs. qPCRs

FIGURE 1. MHCII+ B cells prime naive CD4+ T cells inefficiently. A total of 2 × 10⁶ CFSE-labeled OT-II cells were transferred to WT, B-MHCII, and MHCII KO mice. Mice were immunized s.c. with 200 μg OVA emulsified in CFA (A-C) or i.v. with 100 μg OVA 323–339 peptides and 75 μg LPS (D-F). (A) Proliferation of CD19+ TCRβ+ OT-II cells in draining LNs 4 d after s.c. immunization with OVA/CFA. (B) Total number of and (C) mean fluorescence intensity of CD44 on OT-II cells in draining LNs of WT, BMHCII, and MHCII KO mice 4 d after OVA/CFA immunization. (D) Proliferation of CD19+ TCRβ+ OT-II cells in the spleen 4 d after OVA peptide immunization. (E) Total number of and (F) mean fluorescence intensity of CD44 on splenic OT-II cells. Bar graphs in (B), (C), (E), and (F) show mean ± SEM. n = 3–5 mice/group, representative of two to three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, calculated using Student t test (B and C) or one-way ANOVA with Tukey’s analysis (E and F).
were performed on a 7500 Real Time PCR system machine (Applied Biosystems). Data were analyzed using the \( \Delta \Delta \) cycle threshold method.

**ELISPOT and ELISA assays**

For NP-specific ELISPOTS, splenocytes were incubated on 10 μg/ml NP5-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-ImmuNoSpot 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 in which the MHCII, Ab^b, locus is targeted with a “conditional gene repair” cassette (50), permitting expression of MHCII in any one cell type to which Cre has been targeted (46). In these mice, designated as MHCII Ab^b STOP/STOP, the Ab^b gene (which is targeted in traditional MHCII KO mouse strains) (47) is silenced by insertion of a transcriptional STOP cassette (50) flanked by LoxP sites into intron 1. This allows MHCII Ab^b to be activated under the control of its own promoter and regulatory elements after Cre-mediated recombination and cassette deletion. In the absence of Cre, MHCII Ab^b STOP/STOP mice phenotypically resemble MHCII Ab^b/— mice with no I-A^b expression and no conventional CD4+ T cells in the thymus or periphery (46). To generate mice in which MHCII is restricted to B cells, we crossed CD19 Cre/Cre mice (51) on a heterozygous background for MHCII (MHCII+/—) (47), to MHCII Ab^b STOP/STOP mice to generate pups that were MHCII Ab^b STOP/STOP CD19Cre/+ (referred to as B-MHCII mice). WT control MHCII STOP/+ CD19 Cre/+ mice had one WT allele of Ab^b and, therefore, expressed MHCII on all APC subsets.

Approximately 97% of B220+ TCRβ^+ B cells expressed MHCII in the spleen and lymph nodes (LNs) of WT mice (Supplemental Fig. 1A) (46), whereas ~90–95% of B cells expressed MHCII in B-MHCII mice. All subsets of CD19^+ B cells examined expressed MHCII in B-MHCII mice, and there was no preferential MHCII expression in any one subset (46). All non–B cell APC populations including DCs and macrophages in B-MHCII mice were MHCII−, whereas they were MHCII+ in WT mice (Supplemental Fig. 2). To verify that B cells from B-MHCII mice were indeed transcribing MHCII, we sorted CD19^+ B220^+ B cells from spleens of WT, B-MHCII, and MHCII KO mice, and performed RT-PCR for the targeted Ab^b gene. B cells from B-MHCII mice expressed less MHCII, Ab^b mRNA than did B cells from WT mice (Supplemental Fig. 1B), consistent with the heterozygous genotype of the B cells in the B-MHCII mice. To confirm MHCII Ab^b transcription was restricted to B cells in B-MHCII mice, we sorted TCRβ^+ CD19^− splenocytes, a population that contains all non-B cell APC subsets, from each line of mice. Expression of Ab^b in this non-T/B cell population was equivalent in B-MHCII and MHCII Ab^b STOP/STOP mice (Supplemental Fig. 1C). Targeting the Ab^b gene does not disrupt B cell development as we recently showed that the populations of developing B cells are comparable in the bone marrow (BM) of MHCII Ab^b mice (46). In...
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 T<sub>FH</sub> and GC formation after peptide or protein immunization

We considered the possibility that B cells could interact with T cells to induce T<sub>FH</sub> 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. withhaptenated NP-OVA in alum, which elicits strong GC and Ab responses. Differentiation of OT-II T<sub>FH</sub> 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+ T<sub>FH</sub> on either day 7 or day 14 p.i. (data not shown). In the absence of T<sub>FH</sub>, 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 x 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<sup>+</sup> 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 (B) and (C) show mean ± SEM. n = 3–4 mice/group, representative of three independent experiments.](http://www.jimmunol.org/issueOF.)
B cell priming after i.p. NP-OVA immunization induced much less OT-II cell proliferation than did i.v. immunization with OVA peptide in LPS. We therefore examined T\textsubscript{FH} differentiation in B-MHCII mice after peptide immunization as the increased T cell priming and proliferation might be more conducive to T\textsubscript{FH} differentiation. Although i.v. peptide immunization did induce some CXCR5 expression on OT-II in both WT and B-MHCII mice, there was only a small population of CXCR5\textsuperscript{+} PD-1\textsuperscript{+} T\textsubscript{FH}-like cells present in either strain (Fig. 3A). However, there was no induction of either Bcl6 or IL-21 expression in CXCR5\textsuperscript{+} OT-II cells from either WT or B-MHCII mice p.i., indicating that i.v. peptide immunization does not induce T\textsubscript{FH} responses (Fig. 3B, 3C).

B cell–restricted Ag presentation induces T\textsubscript{FH} 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\textsuperscript{+} T cell compartment of B-MHCII mice with 10\textsuperscript{7} polyclonal CD4\textsuperscript{+} T cells and transferred 1 \times 10\textsuperscript{6} LCMV GP61–80 specific SmartaTCR 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 littermates 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 T\textsubscript{FH} phenotype (Fig. 4A, 4C). T\textsubscript{FH} 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\textsuperscript{+} cells primed only by B cells were indeed T\textsubscript{FH} cells. Overall, these data demonstrate that in the setting of acute viral infection, B cells can induce partial T\textsubscript{FH} differentiation and skew T cells almost exclusively toward the T\textsubscript{FH} lineage.

Because T\textsubscript{FH} 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\textsuperscript{+} IgD\textsuperscript{lo} 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 IgD\textsuperscript{lo} GL-7\textsuperscript{+} 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 T\textsubscript{FH} 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 T\textsubscript{FH} differentiation and GC development after protein immunization

Previous work has shown that generation of a partially differentiated T\textsubscript{FH} cell (pre-T\textsubscript{FH}) (36) is initiated by MHCII\textsuperscript{+} DCs before cognate T–B interactions (31, 34–36). We and others have proposed that B cell Ag presentation completes the T\textsubscript{FH} program (21, 36). However, the ability of MHCII\textsuperscript{+} B cells to complete T\textsubscript{FH} differentiation has not been directly examined. We therefore crossed B-MHCII mice to mice in which only CD11c\textsuperscript{hi} lymphoid-resident DCs are MHCII\textsuperscript{+} (DC-MHCII, referred to as CD11c/Aq\textsuperscript{b}) (6, 36), to generate mice in which MHCII is expressed by conventional DCs and B cells together (B/DC-MHCII mice). To examine T\textsubscript{FH} differentiation in the presence of DC and B cell MHCII expression, we again analyzed transferred OT-II...
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-1hiTfh population found in WT mice (Fig. 5B, 5D); however, PD-1hiTfh 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 $2 \times 10^5$ PFU LCMV Armstrong. Smarta cells had expanded equivalently in WT and B/DC-MHCII mice on day 8 post infection (Fig. 7A), and similar numbers of Smarta cells had differentiated into CXCR5+ PD-1hiBcl6+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 IgG1+ LCMV-specific Abs than did...
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 reconstructing 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 TFH and GCs post-viral infection, in contrast with MHCII+ B cells alone.

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

Follicular regulatory T cells (TFR) express Foxp3 and Bcl6, and localize to the GC to limit the humoral response mediated by TFH and GCs. B/DC-MHCII mice receive 1 × 10⁵ OT-II cells from C57/BL6 mice 7 d before infection to reconstitute the CD4+ T cell compartment. A total of 1 × 10⁴ SMARTA transgenic CD4+ T cells was transferred to WT and B/DC-MHCII mice, and the mice were infected with LCMV Armstrong 1 d later. Splenocytes were analyzed on day 8 postinfection. (A) Total number of SMARTA cells per spleen in WT and B/DC-MHCII mice on day 8 postinfection (gated on CD19-TCRβ+CD45.1+ cells). Numbers represent the percent of Smarta cells that are CXCR5+PD-1+. (B) Representative FACS plots of CD19-TCRβ+CXCR5+CXCR5- Smarta cells for PD-1 and CXCR5 expression. (C) Total number of PD-1⁺ CXCR5⁺ Smarta TFH per spleen in WT and B/DC-MHCII mice on day 8 p.i., quantified from the plots in (A). (D) Histogram overlay of Bcl6 expression of CXCR5⁺PD-1⁺ T FH from the plots shown in (A). WT are shown with the black line and B/DC-MHCII by the dashed line. (E) Representative FACS plots of GC B cells on day 8 postinfection (gated on CD19⁺ B220⁺ F4/80⁻ GR-1⁻ TCRβ⁻ cells). Numbers represent the percent of B cells that are GCs. (F) Measurement of LCMV-specific IgG in the serum of WT and B/DC-MHCII mice on day 8 p.i., compared with uninfected C57/BL6 mice. Bar graphs in (C) and (F) show mean ± SEM. Data are representative of two independent experiments with four to five mice per group. **p < 0.01, ***p < 0.001, calculated using a one-way ANOVA with Tukey’s analysis.
Bcl6+ T<sub>FH</sub> cells (55–57). T<sub>FR</sub> numbers increase during later stages of the GC response, suggesting that T<sub>FR</sub> regulate the GC as the immune response progresses (55). T<sub>FR</sub> cells limit the size of the GC response, as well as maintaining the production of Ag-specific Abs (55, 57). OT-II cells do not become T<sub>FR</sub> cells p.i. (see below) (55, 57), and T<sub>FR</sub> may differentiate from naturally occurring thymically derived Foxp3<sup>+</sup> regulatory T cells (Tregs). As we previously noted, B/DC-MHCII mice lack thymic selection of CD4<sup>+</sup> T cells and, therefore, also lack functional Tregs (data not shown) and provide a model to study the GC response in the absence of T<sub>FR</sub>.

At day 7 p.i. with NP-OVA, B/DC-MHCII mice have a comparable GC response to WT mice (Fig. 6A), suggesting that Tregs and T<sub>FR</sub> do not impact the early stages of the GC response. However, on day 14 p.i., B/DC-MHCII mice had at least twice the number of splenic Fas<sup>+</sup> GC B cells as did WT mice (Fig. 8A, 8B). In parallel, B/DC-MHCII mice also had increased numbers of OT-II T cells; the numbers of OT-II T<sub>FH</sub> 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<sub>FH</sub> (Fig. 8D). Although B/DC-MHCII and WT spleens contained a similar number of Ag-specific NP<sup>+</sup> GC B cells, B/DC-MHCII mice also had a large number of NP<sup>+</sup> IgG1<sup>+</sup> GC B cells (Fig. 8A). Thus, the ratio of NP-binding to NP-negative cells within the IgG1<sup>+</sup> 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<sup>+</sup> T cells and Tregs.

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

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**FIGURE 8.** Increased OT-II and GC responses in the absence of endogenous CD4<sup>+</sup> T cells. A total of 1 × 10<sup>7</sup> 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<sup>+</sup>B220<sup>+</sup> splenocytes, top plots), IgG1 expression, and NP-specific cells of the GC population (gated on CD19<sup>+</sup>B220<sup>+</sup>Fas<sup>+</sup>IgD<sub>lo</sub> 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<sup>+</sup>IgG1<sup>+</sup> (bottom plots). (B) Total number of NP-specific IgG1<sup>+</sup> GCs on day 14 p.i. as quantified from the plots in (A). (C) Ratio of the percentage of NP<sup>+</sup> to NP<sup>−</sup> cells of CD19<sup>+</sup>B220<sup>+</sup>Fas<sup>+</sup>IgD<sub>lo</sub>IgG1<sup>+</sup> GC B cells. (D) Representative FACS plots of CD19<sup>+</sup> TCR<beta><sup>+</sup> OT-II cells (top plots) and CCR5<sup>+</sup>PD-1<sup>hi</sup> OT-II T<sub>FH</sub> (bottom plots) on day 14 p.i. Numbers represent the percent of CD4<sup>+</sup> T cells that are OT-II (top plots) and the percent of OT-II cells that are CCR5<sup>+</sup>PD-1<sup>hi</sup> (bottom plots). (E) Total number of splenic CD19<sup>+</sup> TCR<beta><sup>+</sup> OT-II cells on day 14 p.i. as quantified from the 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 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 TFH or a GC response. However, in response to acute viral infection, B cell Ag presentation skews the Ag-specific T cell response toward the TFH subset. Nevertheless, MHCII expression restricted to DCs and B cell mediates optimal TFH differentiation and expansion, as well as GC formation with affinity maturation and isotype switching of Ag-specific Abs 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 TFH 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 TFH 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-TFH in mice with MHCII Ag presentation restricted to DCs (36). Multiple recent investigations have examined the requirement for B cells in the differentiation of TFH. Earlier studies had demonstrated that mice lacking B cells or the ability to maintain T-B conjugates lack TFH (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 TFH differentiation (30, 31).

These data suggest that DCs and B cells may provide qualitatively distinct signals to T cells that contribute to TFH differentiation.
For example, IL-6, presumably produced by DCs, has an in vitro role in the induction of BC6 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 cell populations can be rectified by reconstituting a polyclonal CD4+ T cell population (which includes Foxp3+ Tregs) or by adding back only Foxp3+ Tregs. Because an overabundance 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 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