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B cell antigen presentation in the initiation of Follicular Helper T cell and Germinal Center differentiation

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Abstract

High affinity class-switched antibodies and memory B cells are products of the germinal center. The $CD4^+$ T cell help required for the development and maintenance of the germinal center is delivered by follicular helper T cells (T_{FH}), a $CD4^+$ helper T 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-dependent DC antigen 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. Here we examine the contributions of MHCII antigen presentation by B cells to T_{FH} differentiation and GC responses in several contexts. B cells alone do not efficiently prime naïve $CD4^+$ T cells or induce T_{FH} following protein immunization; however, during LCMV infection B cells induce T_{FH} differentiation despite the lack of effector $CD4^+$ T cell generation. Still, MHCII-positive DCs and B cells cooperate for optimal T_{FH} and GC B cell differentiation in response to both model antigens 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 germinal center response.

Introduction

$CD4^+$ T cells play a central role in immune responses, both as effector cells and by providing help to other cells, including B cells. Naïve $CD4^+$ T cells must be activated by antigen presenting cells (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-positive dendritic cells (DCs) are uniquely positioned to activate naïve $CD4^+$ 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-positive B cells to present antigen 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 cells priming was independent of B cells (17–20). However, studies to directly test the sufficiency of B cell antigen presentation in $CD4^+$ T cell priming are lacking.

Primed $CD4^+$ T cells differentiate into multiple effector subsets, including follicular helper T 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 Bcl6, 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 co-stimulatory molecules, including CD40L, ICOS and PD-1 (21, 30, 31)and cytokines, especially IL-21 and IL-4 (32, 33), that contribute to the formation and function of the germinal center. As 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, prior to $CD4^+$ 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 Bcl6 (36). Pre-T_{FH} lack expression of PD-1 and do not produce significant quantities of the cytokine, 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 antigen-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} 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_{FH} differentiation simply requires persistent TCR signals. Concretely delineating the requirement for individual $MHCII^+$ APCs to initiate and maintain T_{FH} differentiation and development of the germinal center should resolve these conflicts.

In this study, we describe a novel mouse model in which MHCII, I-A^b, is restricted to B cells. We define the ability of B cells to prime naïve $CD4^+$ T cells *in vivo* and the contribution of B cells to T_{FH} differentiation in different contexts. MHCII expression restricted to B cells cannot drive $CD4^+$ T cell priming, T_{FH} 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 antigen specific $CD4^+$ T cells with a T_{FH} phenotype. However, the generation of functional antigen-specific germinal centers 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 T_{FH} program; however, MHCII-dependent antigen presentation by both DCs and B cells is necessary to induce optimal differentiation of T_{FH} and germinal centers.

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/A_β^b mice (6) were bred in house. MHCII $A_\beta^{b\ STOP/STOP}$ mice were developed at Washington University in St. Louis as described and subsequently bred in house (46). B-MHCII mice were breed as $CD19^{Cre/+}$ MHCII $A_\beta^{b\ STOP/-}$, B/DC-MHCII mice additionally had the CD11c/A_β^b transgene. WT control mice were breed as MHCII $^{STOP/+}$ CD19 $^{Cre/+}$. MHCII alleles are denoted in the text as follows: WT as +, MHCII A_β^b KO (47) as - and MHCII $A_\beta^{b\ STOP}$ as STOP. Mice were housed under pathogen free conditions, in accordance with the University of Pennsylvania Animal Care and Use Guidelines and used at 8–18 weeks 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 $Fc\gamma RII^+$ cells and labeled with CFSE where indicated, as previously described (48). OT-II cells were transferred i.v. 1 day prior to i.p. immunization with 50μg NP_{14}-OVA (4–Hydroxy-3-nitrophenylacetyl coupled to ovalbumin) (Biosearch Technologies) in alum (Sigma) as previously described (32, 36). 1×10^4 Smarta cells were transferred i.v. 1 day prior to infection with 2×10^5 PFU LCMV Armstrong (experiments shown in figure 4) or 2×10^4 PFU Armstrong (experiments shown in figure 6) as previously described (49). Virus was grown and titered as described (49). B-MHCII and B/DC-MHCII mice infected with LCMV Armstrong also received ~10^7 $CD4^+$ polyclonal T cells isolated from C57/BL6 mice 7–14 days before infection to reconstitute the $CD4^+$ T cell compartment.

In vitro cultures

Sorted B cells were incubated overnight with 10 μg LPS and one day later were incubated with CFSE labeled purified OT-II cells (see immunizations and infections section above), at a ratio of 1:10, with 10,000 B cells and 100,000 OT-II cells per well, in OVA protein at a concentration of 100μg/mL. CFSE dilution of OT-II cells was analyzed 4 or 5 days later.

Flow cytometry and cell sorting

All antibodies 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 was analyzed using FlowJo software (TreeStar). All FACS plots shown were gated on live, singlet cells.

QPCR

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_{FH} cell qPCR, 18s was used as a housekeeping gene for all other qPCRs. qPCRs were performed on an 7500 Real Time PCR system machine (Applied Biosystems). Data were analyzed using the ΔΔct method.

ELISPOT and ELISA assays

For NP specific ELISPOTS, splenocytes were incubated on 10 ug/ml of NP5-BSA (high affinity) or NP25-BSA (all affinities) (Biosearch Technologies) coated plates (Millipore) and incubated with biotin-anti-mouse-IgG1or 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 antibodies were detected in serum by ELISA. Lysate from baby hamster kidney (BHK) cells infected with LCMV Armstrong was used to coat ELISA plates. HRP-linked antibodies against mouse IgG were used to detect the LCMV reactive antibodies. 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

In order to better study the requirements for various $MHCII^+$ APCs in $CD4^+$ T cell activation, we have recently developed a new mouse strain in which the MHC class II, $A\beta^b$, locus is targeted with a “conditional gene repair” cassette (50), permitting expression of MHCII in any cell type to which Cre has been targeted (46). In these mice, designated as MHCII $A_\beta{}^{b\ STOP/STOP}$, the $A_\beta{}^b$ gene (which is targeted in traditional MHCII knockout mouse strains (47)) is silenced by insertion of a transcriptional STOP cassette (50) flanked by LoxP sites into Intron 1. This allows MHCII $A_\beta{}^b$ 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 $A_\beta{}^{b\ STOP/STOP}$ mice phenotypically resemble MHCII $A_\beta{}^{b\ -/-}$ 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 $A_\beta{}^{b\ STOP/STOP}$ mice in order to generate pups that were MHCII $A_\beta{}^{b\ STOP/-}$ $CD19^{Cre/+}$ (referred to as B-MHCII mice). WT control MHCII $^{STOP/+}$ CD19 $^{Cre/+}$ mice had one WT allele of $A_\beta{}^b$ and therefore expressed MHCII on all APC subsets.

Approximately 97% of $B220^+$ $TCR\beta^-$ B cells expressed MHCII in the spleen and LNs of WT mice (Supplemental Figure 1A, 46), while approximately 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 negative, whereas they were MHCII positive in WT mice (Supplemental Figure 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 $A_\beta{}^b$ gene. B cells from B-MHCII mice expressed less MHCII, $A\beta^b$, mRNA than did B cells from WT mice (Supplemental Figure 1B), consistent with the heterozygous genotype of the B cells in the B-MHCII mice. To confirm MHCII, $A\beta^b$, transcription in B-MHCII mice was restricted to B cells, $TCR\beta^-$ $CD19^-$ splenocytes, a population that contains all non-B cell APC subsets, were sorted from each line of mice. Expression of $A\beta^b$ in this non-T/B cell population was equivalent in B-MHCII and MHCII $A_\beta{}^{b\ STOP/STOP}$ mice (Supplemental Figure 1C). Targeting the $A\beta^b$ gene does not disrupt B cell development as we recently showed that the populations of developing B cells are comparable in the BM of MHCII $A_\beta{}^{b\ STOP/STOP}$, B-MHCII and WT mice (46). Additionally, 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 naïve $CD4^+$ 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 ovalbumin (OVA) protein and incubated with CFSE labeled ovalbumin-specific TCR transgenic OT-II cells for 4 days. 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 Figure 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 antigen to activate naïve $CD4^+$ T cells. Additionally, WT and B-MHCII B cells induce comparable $CD4^+$ T cell proliferation *in vitro* despite expressing different levels of MHCII.

B cells prime naïve CD4 T cells poorly in response to nominal protein antigen in vivo

Although B cells are the most numerous MHCII-positive APC in secondary lymphoid tissues, their contribution to the priming of naïve $CD4^+$ T cells *in vivo* remains unclear. The B-MHCII mice provide the ideal system to examine this question. Thymic cortical epithelium is MHCII-negative 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 antigen-specific TCR transgenic $CD4^+$ T cells. CFSE labeled OT-II cells were transferred into MHCII-deficient, B-MHCII, or WT recipients 1 day prior to s.c. immunization with OVA protein emulsified in CFA. Four days after immunization, 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 (Figure 1A, B). 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 (Figure 1C).

Utilizing a protein immunization system limits antigen delivery to the small number of B cells with a B cell receptor specific for the immunizing antigen (53), and non-BCR mediated antigen 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, mice were immunized IV with OVA 323–339 peptide and LPS. OT-II cells in WT mice exhibited extensive proliferation, with most of the cells found in the 4^{th} division or greater (Figure 1D). In contrast, OT-II cell proliferation induced by B cells alone in B-MHCII mice was sub-optimal as the majority of cells had divided only once or twice (Figure 1D). OT-II cells primed by B cells did have increased CD44 expression in comparison to mice that were not immunized; although, they expressed much less CD44 than OT-II cells primed in WT mice (Figure 1F) and produced significantly interferon gamma and IL-2 (Supplemental Figure 3). However, there was no increase in the number of OT-II cells in either the spleen or peripheral LNs (Figure 1E) of immunized B-MHCII mice compared to unimmunized mice. Thus, B cells are capable of inducing minimal $CD4^+$ T cell priming *in vivo* when directly targeted with processed antigen, but B cell antigen presentation alone does not induce the activation and expansion observed when other $MHCII^+$ APC populations are also functional.

B cell restricted antigen presentation is not sufficient to elicit T_{FH} and GC formation following peptide or protein immunization

We considered the possibility that B cells could interact with T cells to induce T_{FH} and germinal center 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 after immunization with haptenated NP-OVA in alum, which elicits strong germinal center and antibody responses. Differentiation of OT-II T_{FH} and GC B cells were examined 7 and 14 days after immunization. Similar to i.v. immunizations, OT-II cells in B-MHCII mice underwent minimal proliferation and no expansion after NP-OVA immunization (Figure 2A, C). Additionally, up-regulation of CXCR5 was impaired and there was no differentiation of $CXCR5^+$ $PD\text{-}1^+$ T_{FH} differentiation on either day 7 (Figure 2B) or d14 post immunization (data not shown). In the absence of T_{FH}, neither antigen-specific germinal centers (Figure 2D, 2F) nor high affinity plasma cells (PCs) in the spleen (Figure 2E) or BM (data not shown) were formed. Importantly, WT and B-MHCII mice had comparable numbers of IgM PC responses in the spleen on d7 (data not shown) and d14 post immunization (Figure 2G), in agreement with previous data suggesting that the early IgM PC response is T cell independent (52). Together, these data show that B cell restricted antigen presentation induces neither T_{FH} nor GC responses after protein immunization.

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_{FH} differentiation in B-MHCII mice after peptide immunization as the increased T cell priming and proliferation might be more conducive to T_{FH} differentiation. While 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 $CXCR5^+$ $PD\text{-}1^+$ T_{FH}-like cells present in either strain (Figure 3A). However, there was no induction of either Bcl6 or IL-21 expression in $CXCR5^+$ OT-II cells from either WT or B-MHCII mice after immunization, indicating that i.v. peptide immunization does not induce T_{FH} responses (Figure 3B, C).

B cell restricted antigen presentation induces T_{FH} differentiation following viral infection

Immunization with model antigens in adjuvant is a useful tool for understanding the biology of an immune response, but does not always mimic the processes that occur in the context of infection. To examine B cell restricted antigen presentation during acute viral infection, we reconstituted the $CD4^+$ T cell comparment of B-MHCII mice with 10^7 polyclonal $CD4^+$ T cells and transfered 1×10^4 LCMV GP61–80 specific SmartaTCR Tg T cells to WT and B-MHCII mice one day prior to infection with LCMV Armstrong. On day 8 post infection, there was much less expansion of Smarta T cells in infected B-MHCII mice than in WT littermates with approximately 100x fewer cells (Figure 4C). However, Smarta cells did not expand in infected MHCII KO mice; thus, the expansion observed in B-MHCII mice was antigen-specific. Strikingly, upwards of 90% of the Smarta cells in B-MHCII spleens exhibited a T_{FH} phenotype (Figure 4A), (Figure 4C). T_{FH} cells primed in WT and B-MHCII mice had equivalent levels of Bcl-6 mRNA (data not shown) and protein (Figure 4D), and also expressed equivalent levels of IL-21 mRNA (Figure 4E), suggesting that the $CXCR5^+$ cells primed only by B cells were indeed T_{FH} cells. Overall, these data demonstrate that in the setting of acute viral infection, B cells can induce partial T_{FH} differentaion, and skew T cells almost exclusively toward the T_{FH} lineage.

As T_{FH} play a critical role in the GC B cell reponse, we next asked if LCMV-specific GC responses were present in LCMV-infected B-MHCII mice. As there are no reagents to asses LCMV specific B cells by FACS, we quantified the number of $GL\text{-}7^+$ IgD^{lo} B cells in spleens of WT and B/DC-MHCII mice by FACS and measured serum IgG antibodies by ELISA on day 8 post infection.After infection, WT mice generated significant numbers of IgD^{lo} $GL\text{-}7^+$ GC B cells; however, B-MHCII mice had almost no GC B cells, close to the background level observed in uninfected mice (Figure 4F). Consistent with these data, B-MHCII mice generated only minimal LCMV specific IgG^+ antibody titers, though greater then the levels in uninfected mice (Figure 4G). Thus, the small number of T_{FH} cells generated in B-MHCII mice after LCMV infection were insufficient for GC formation.

The combination of DC and B cell antigen presentation is sufficient for T_{FH} differentiation and GC development after protein immunization

Previous work has shown that generation of a partially differentiated T_{FH} cell (pre-T_{FH}) (36) is initiated by MHCII-positive DCs prior to cognate T-B interactions (31, 34–36). We and others have proposed that B cell antigen presentation completes the T_{FH} program (21, 36). However, the ability of MHCII-positive B cells to complete T_{FH} 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 $CD11c/A_\beta{}^b$ (6, 36)], to generate mice in which MHCII is expressed by conventional DCs and B cells together (B/DC-MHCII mice). To examine T_{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 (Figure 5A) and generated similar numbers of $CXCR5^+$ OT-II cells with equivalent expression of Bcl6 mRNA and protein (Figure 5B, C, E, F). Consistent with our prior work, antigen-specific $CD4^+$ T cells primed by DCs alone lack the PD-1^{hi} T_{FH} population found in WT mice (Figure 5B, D); however, PD-1^{hi} T_{FH} are restored in B/DC-MHCII mice (Figure 5B, D). While $CXCR5^+$ OT-II cells primed only by DCs exhibit approximately a 10 fold reduction in IL-21 mRNA levels when compared to WT-T_{FH}, T_{FH} primed by both DCs and B cells exhibit similar levels of IL-21 transcript as WT-T_{FH} (Figure 5G). Together these data demonstrate that MHCII-positive DCs and B cells cooperate for T_{FH} differentiation after immunization, as neither population alone is sufficient for T_{FH} differentiation but the combination is.

As T_{FH} function to drive and sustain the GC B cell response, we hypothesized that the combination of DC and B cell antigen would also suffice for differentiation of GC B cells. Indeed, seven days after immunization, both WT and B/DC-MHCII spleens contained equivalent numbers of Fas^+ IgD^{lo} NP-binding, IgG^{1+} GC B cells (Figure 6A, B). Germinal centers function to generate high affinity class-switched plasma cells and memory B cells. Fourteen days after NP-OVA immunization there were similar numbers of high affinity $IgG1^+$ NP^+ ASCs in the spleen (Figure 6C), as well as in the BM (data not shown) of WT and B/DC-MHCII mice. Similarly, on day 14 post-immunization (data not shown), as well as day 29 post immunization (Figure 6D, E) 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 germinal center B cell differentiation after protein immunization.

DC and B cell antigen presentation during viral infection

As B cell priming alone was insufficient to induce optimal T_{FH} or antibody responses following acute LCMV infection, we hypothesized that the addition of DC antigen 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 post infection (Figure 7A) and similar numbers of Smarta cells had differentiated into $CXCR5^+$ PD-1^{hi} Bcl6+T_{FH} cells in B/DC-MHCII and WT mice (Figure 7B, C, D), indicating that DC and B cell MHCII expression is sufficient for T_{FH} differentiation in the setting of viral infection.

As DC and B cell MHCII expression was sufficient for antigen specific GC B cell responses to immunzation, we asked if this was also true following viral infection. While GC B cells did develop in B/DC-MHCII mice, the GC population was significantly smaller than in WT mice (Figure 7E). In agreement, B/DC-MHCII mice generated lower titers of IgG^+ LCMV-specific antibodies than did WT mice, although the levels were significantly greater than those of uninfected mice (Figure 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-positive DCs and B cells do generate both T_{FH} and GCs following viral infection, in contrast to MHCII+ B cells alone.

B/DC-MHCII mice have increased GCs in the absence of peripheral Tregs

Follicular regulatory T cells (T_{FR}) express Foxp3 and Bcl6 and localize to the GC to limit the humoral response mediated by Bcl6-positive T_{FH} cells (55–57). T_{FR} numbers increase during later stages of the GC response, suggesting that T_{FR}s regulate the GC as the immune response progresses (55). T_{FR} cells limit the size of the GC response, as well as, maintaining the production of antigen-specific antibodies (55, 57). OT-II cells do not become T_{FR} cells after immunization (Figure 9F, (55, 57)) and T_{FR} may differentiate from thymically-derived $Foxp3^+$ nTregs. As we previously noted, B/DC-MHCII mice lack thymic selection of $CD4^+$ 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_{FR}.

At day 7 p.i. with NP-OVA, B/DC-MHCII mice have a comparable GC response to WT mice (Figure 6A), suggesting that Tregs and T_{FR}s 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^+ GC B cells as did WT mice (Figure 8A, B). In parallel, B/DC-MHCII mice also had increased numbers of OT-II T cells; the numbers of OT-II T_{FH} 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_{FH} (Figure 8D). Although B/DC-MHCII and WT spleens contained a similar number of antigen specific NP^+ GC B cells, B/DC-MHCII mice also had a large number of NP^- $IgG1^+$ GC B cells (Figure 8A). Thus, the ratio of NP-binding to NP-negative cells within the $IgG1^+$ GC population was significantly reduced in B/DC-MHCII mice, (Figure 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 approximately 10–15% $FoxP3^+$ Tregs (58)) which resulted in normalization of the numbers of both OT-II T cells and GC B cells (Figure 9A, B, D). The ratio of NP+ to NP- GC B cells also returned to WT levels (Figure 9C). We hypothesized that the presence of T_{FR}s in the polyclonal $CD4^+$ T cells transferred into B/DC-MHCII mice was responsible for the normalization of the GC response. To directly determine if $Foxp3^+$ T cells could mediate this process, we transferred 5×10^5 $Foxp3^+$ GFP^+ Tregs from WT Foxp3-GFP reporter mice (45) (a number equivalent to approximately 5×10^6 bulk $CD4^+$ T cells) in addition to 1×10^5 OT-II cells and immunized the mice with NP-OVA. On day 14 post-immunization, GC numbers in B/DC-MCHII mice were reduced to the levels of WT in those mice that also received $Foxp3^+$ Tregs (data not shown), though 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 (Figure 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 describing 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

(Figure 8F, (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 germinal center and that MHCII expression by other cell types is not required.

Discussion

In this study we investigated the role for B cell antigen presentation in naïve $CD4^+$ T cell priming, T_{FH} differentiation and development of the GC. We found that MHCII antigen presentation restricted to B cells mediates very inefficient $CD4^+$ T cell priming in response to either nominal protein or peptide antigens, without the induction of either T_{FH} or a GC response. However, in response to acute viral infection, B cell antigen presentation skews the antigen 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 GC formation with affinity maturation and isotype switching of antigen specific antibodies in response to immunization and viral infection. These studies highlight the requirement for cooperation amongst multiple cells during the initiation of a humoral immune response.

The ability of B cells to activate naïve $CD4^+$ T cells has been previously examined with conflicting results. It has been shown that B cells are poor $CD4^+$ activators (19) and may tolerize $CD4^+$ 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 as they are the most numerous professional APC in secondary lymphoid tissues. The inability of B cells to prime naïve $CD4^+$ T cells after immunization may reflect the absence of an appropriate combination of co-stimulatory molecules and inflammatory cytokines expressed by DCs or may simply be a problem of anatomy as 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 naïve, antigen specific B cells and permit them to interact with antigen specific T cells (61). Thus, the reasons for the inability of B cells to effectively prime naïve $CD4^+$ T cells are not clear but may be a combination of location and signal quality.

Our data demonstrate that antigen presentation by DCs and B cells together is sufficient for optimal T_{FH} differentiation in multiple settings, though the role of B cell antigen presentation in the process may be different following immunization and in response to infection. Recent studies have demonstrated that the differentiation of T_{FH} precursors requires DCs and is initiated prior to 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 antigen 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). Additionally, examination of gene-deficient mice also suggested that B cell expression of the costimulatory molecules, ICOS and PD-L2, were 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. 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 T_{FH} after protein immunization (64–66). However, more work has been done to identify costimulatory molecules expressed by B cells that may affect T_{FH} differentiation. B cells can provide many signals to T_{FH} 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 CD80, are important in T_{FH} and GC B cell differentiation (21, 22, 30, 67). Although it has been suggested that T_{FH} differentiation does not require unique B cell signals but rather sustained antigen presentation (42, 43), most studies support the alternative model that cognate, antigen-specific B cells maintain T_{FH} that differentiate early after DC interactions. The striking observation described here that B cell restricted antigen presentation exclusively primes T_{FH} cells, at the expense of CXCR5 negative effector T cells after viral infection suggests that B cells may express and provide unique signals to T cells to induce the T_{FH} program. B cells alone exclusively generated T_{FH} cells after infection but at greatly reduced numbers. The addition of DC antigen presentation is sufficient to induce optimal antigen specific T cell expansion after infection, as well as restore the normal proportion of T_{FH} and effector T cells. Therefore, DCs drive $CD4^+$ effector T cell differentiation and T cell expansion following infection; whereas, B cell antigen presentation is the force behind T_{FH} differentiation.

The antigen presentation requirements for GC B cell differentiation largely parallel those required for optimal T_{FH} differentiation. Following 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 antigen specific T_{FH} cells after viral infection, they were unable to form GCs and LCMV specific IgG antibody. This may be due to the fact that overall numbers of SMARTA T_{FH} were greatly reduced in B-MHCII mice compared to WT mice. The addition of DC antigen presentation was able to induce some GCs and antigen 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 antibodies 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 antibody responses may be context-dependent.

Finally, previous studies have demonstrated increased germinal center and T cell responses in the absence of follicular regulatory T cells (T_{FR}s) (55–57), and our data also suggest a role for these cells. In the absence of endogenous $CD4^+$ T cells, including nTregs, we observed increased antigen specific T cells in the absence of Tregs, including an increase in T_{FH} cells, associated with increased GCs. Both the overabundance of 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. As an overabundance of T_{FH} cells is linked to autoantibody production (38), T_{FR} 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 T_{FR} cells in other contexts, as well as dissecting the role(s) of DC and B cell antigen presentation in other settings.

The results described here highlight the controlled and cooperative nature of $CD4^+$ T cell activation, T_{FH} 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 naïve $CD4^+$ T cells. Additionally, the stringent requirements for T_{FH} activation may be altered in infection or autoimmunity and perhaps a signal from either a DC or a B cell is sufficient for T_{FH} differentiation. The multiple steps required in T_{FH} differentiation may serve as a checkpoint in the prevention of autoimmunity by ensuring the antigen specificity of responding T_{FH} and ensuring that they make IL-21 only when it is appropriate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this work

DC	dendritic cell
GC	germinal center
MHCII	MHC class II
NP-OVA	(4-hydroxy-3-nitrophenyl) acetyl coupled to OVA
PD-1	programmed cell death 1
T_{FH}	follicular helper T cell
WT	wild type
p.i.	post immunization
LCMV	lymphocytic choriomeningitis virus.

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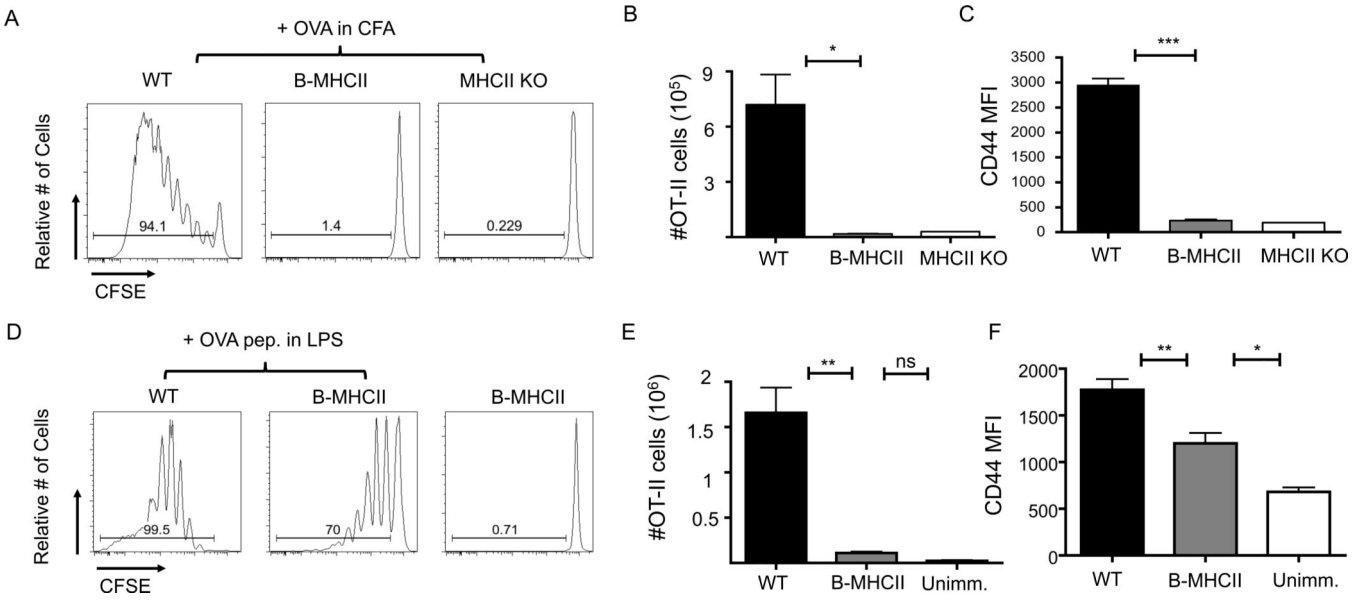

Figure 1.

MHCII$^+$ B cells prime naïve CD4$^+$ T cells very inefficiently. 2×10^6 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 100μg OVA 323–339 peptide and 75μg LPS (D–F). (**A**) Proliferation of CD19$^-$ TCRβ$^+$ OT-II cells in dLNs 4 days after s.c. immunization with OVA/CFA. (**B**) Total number of and (**C**) mean fluorescence intensity of CD44 on OT-II cells in dLNs of WT, BMHCII and MHCII KO mice 4 days after OVA/CFA immunization. (**D**) Proliferation of CD19$^-$ TCRβ$^+$ OT-II cells in the spleen 4 days 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 per group, representative of 2–3 independent experiments. * indicates a p value of <0.05, ** indicates a p value of <0.01 and *** indicates a p value of <0.001, calculated using Student's *t* test (B,C) or one way ANOVA with Tukey's analysis (E,F).

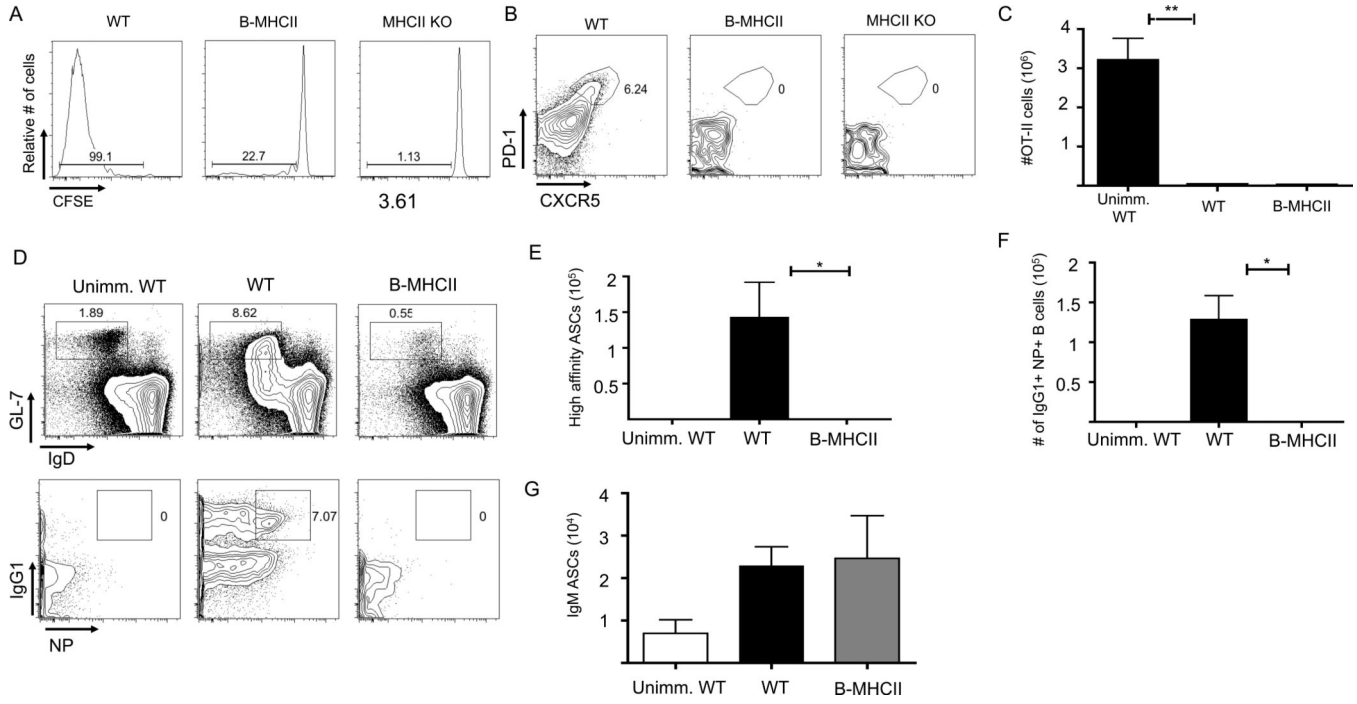

Figure 2.

MHCII dependent antigen presentation by B cells alone does not elicit T_{FH} and GC responses in response to protein immunization. 1×10^6 OT-II cells were 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 diltution of OT-II cells in the spleen on d7 p.i. Gated on $CD19^-$ $TCR\beta^+$ $CD90.1^+$ OT-II cells (**B**) Representative FACS plots of OT-II cells to identify $CXCR5^+PD\text{-}1^{hi}$ T_{FH} on d7 p.i. Numbers indicated percentage of OT-II cells that are $CXCR5^+$ $PD\text{-}1^{hi}$ (**C**) Total number of OT-II T_{FH} in WT and B-MHCII mice on d7 p.i. (**D**) Representative FACS plots of GL-7^+ germinal center B cells (top) and IgG1 expression and NP specific cells of the GC (bottom) on day 7 p.i. Gated on $CD19^+B220^+$ cells (top) and further on GL-7^+ IgD^-. Numbers represent the percentage of B cells that are GCs (top) and percent of GC B cells that are NP^+ $IgG1^+$ (bottom) (**E**) Total number of splenic NP specific, $IgG1^+$ GC B cells on d7 p.i. (**F**) Total number of high affinity, NP specific $IgG1^+$ ASCs per spleen and (**G**) Total number of IgM^+ ASCs per spleen on day 14 p.i. as determined by ELISPOT. Bar graphs in (C), (E), (F) and (G) show mean ± SEM. n=5–6 mice, data are pooled from two independent experiments. * denotes a p value of <0.05 and ** denotes a p value of <0.01, calculated using Student's *t* test.

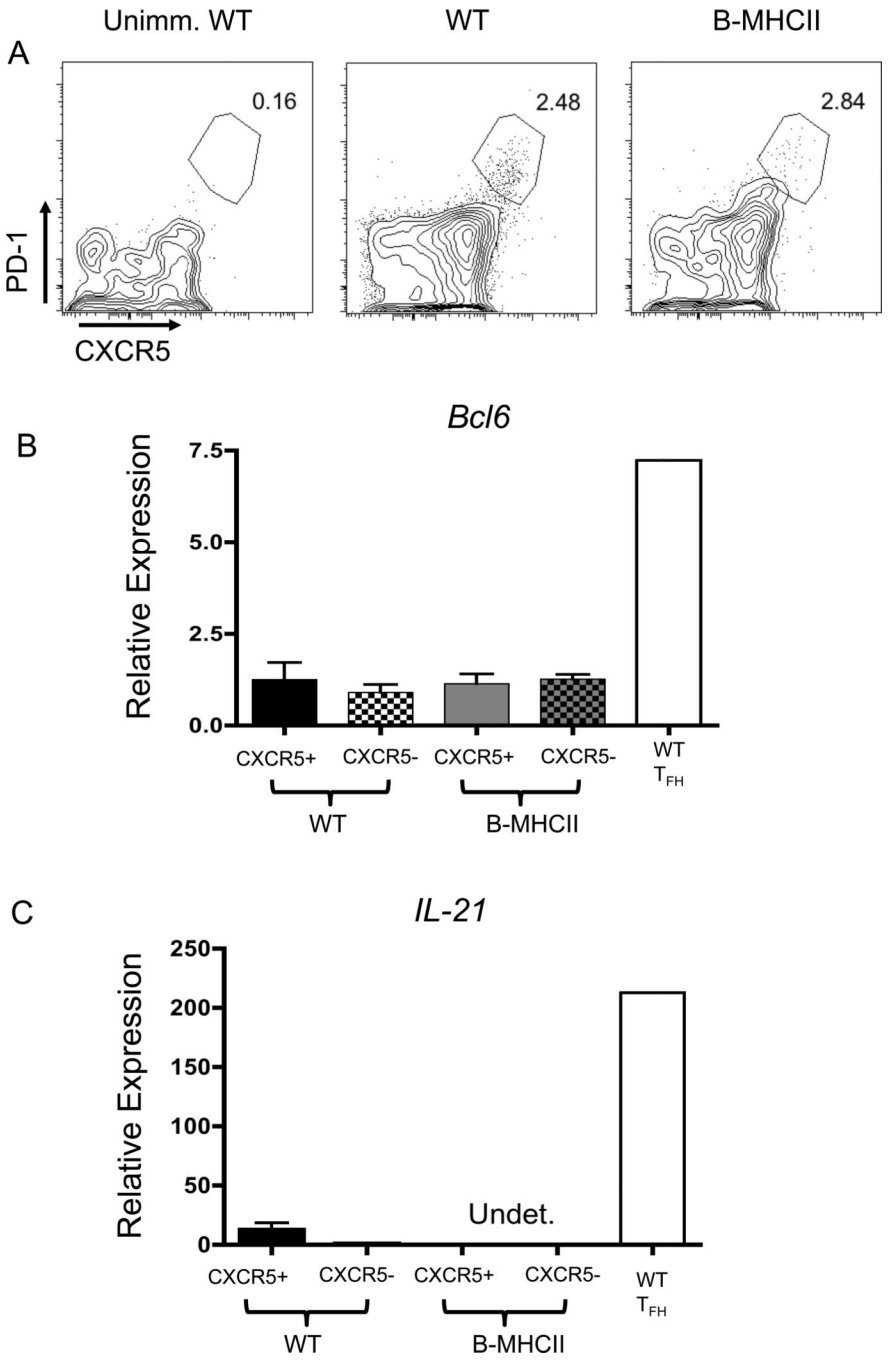

Figure 3.

Minimal T_{FH} differentiation in response to peptide immunization. 2×10^6 CFSE labeled OT-II cells were transferred to WT and B-MHCII mice. Mice were immunized i.v. with 100μg OVA 323–339 peptide and 75μg LPS. Splenocytes were examined 7 days after immunization. (**A**) Representative FACS plots of $CD19^-$ $TCR\beta^+$ $CD4^+$ OT-II cells to identify $CXCR5^+$ $PD\text{-}1^+$ T_{FH} cells. $CXCR5^+$ and $CXCR5^-$ OT-II cells were sorted from WT and B-MHCII mice after immunization and examined for (B) Bcl6 mRNA and (C) IL-21

mRNA. Bar graphs in (**B**) and (**C**) show mean ± SEM. n=3–4 mice per group, representative of 3 independent experiments.

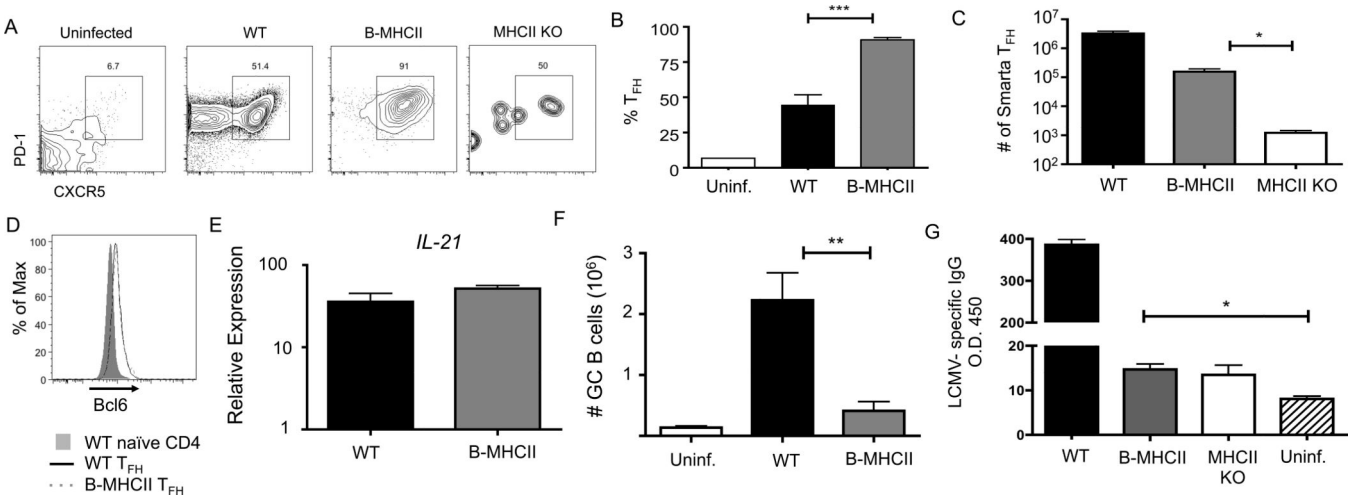

Figure 4.

B cell antigen presentation preferentially drives T_{FH} 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 days prior to infection to reconstitute the $CD4^+$ T cell compartment. 1×10^4 SMARTA transgenic $CD4^+$ T cells were transferred to WT and B-MHCII mice and the mice were infected with LCMV Armstrong one day later. Splenocytes were analyzed on day 8 post infection. (**A**) Representative FACS plots of $CD19^-$ $TCR\beta^+$ $CD4^+$ SMARTA cells to identify $CXCR5^+$ $PD\text{-}1^+$ T_{FH} cells. (**B**) Percentage of SMARTA cells in WT and B-MHCII mice that are $CXCR5^+$ $PD\text{-}1^+$ T_{FH} cells. (**C**) Total number of splenic SMARTA T_{FH} cells in WT, B-MHCII and MHCII KO mice. (**D**) Histogram overlay of Bcl6 expression by $CXCR5^+$ $PD\text{-}1^+$ SMARTA T_{FH} cells. (**E**) Relative expression of IL-21 mRNA in sorted $CXCR5^+$ $PD\text{-}1^+$ SMARTA cells. (**F**) Total number of $CD19^=$ $B220^+$ IgD^{lo} $GL\text{-}7^+$ GC B cells. (**G**) Measurement of LCMV specific IgG in the serum on d8 p.i., compared to uninfected C57/BL6 mice. * denotes a p value of < 0.05, ** denotes a p value of <0.01 and *** denotes a p value of <0.001 calculated using Student's *t* test. Bar graphs show mean ± SEM. Data are representative of 2 independent experiments with 3–6 mice per group.

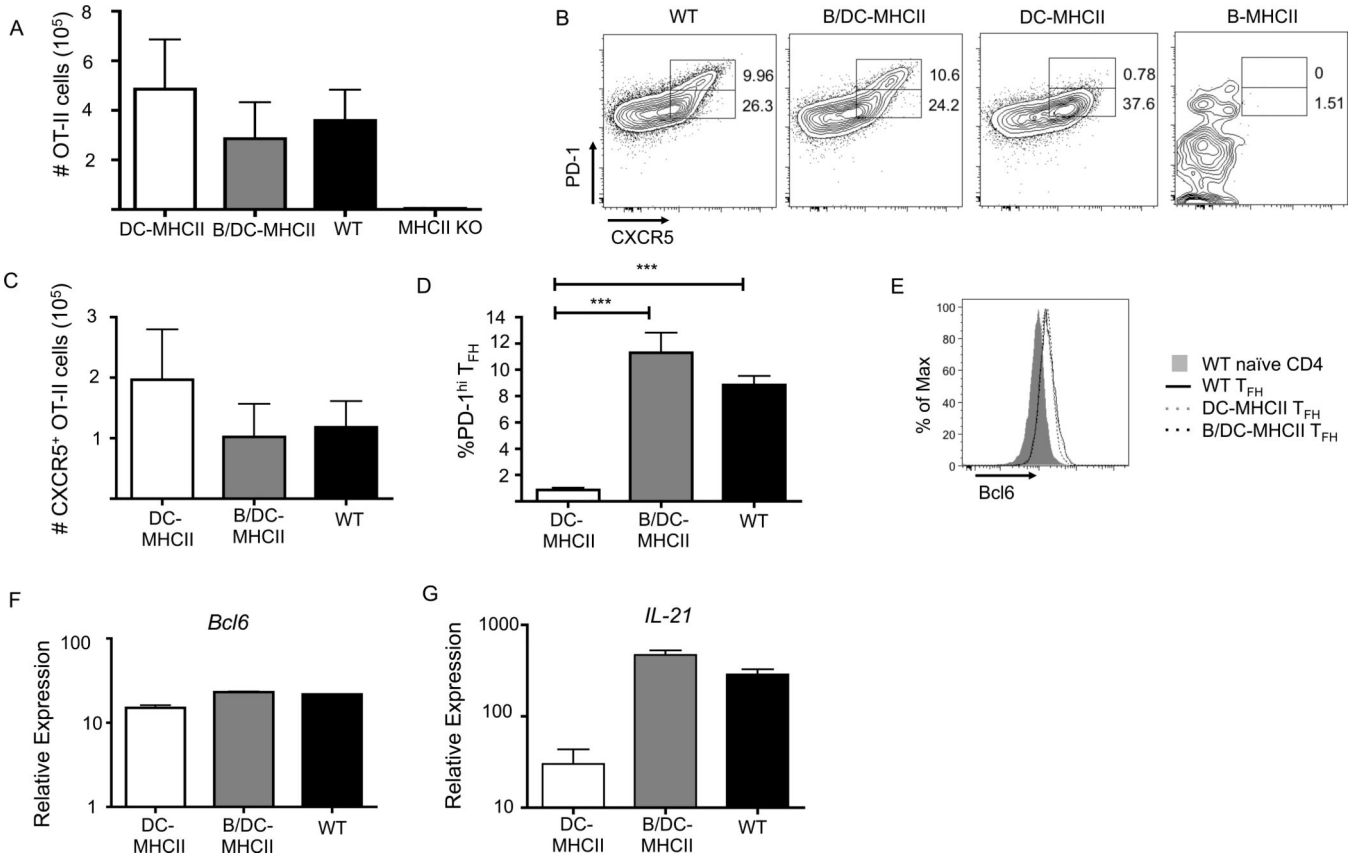

Figure 5.

MHCII antigen presentation by DCs and B cells cooperates for T_{FH} differentiation. 1×10^5 OT-II cells were 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 ($CD19^-$ $TCR\beta^+CD90.1^+$) 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 T_{FH}. Numbers represent the percent of OT-II cells that are $CXCR5^+$ $PD\text{-}1^{hi}$ and $PD\text{-}1^{int}$ (**C**) Total number of $CD62L^-$ $CXCR5^+$ OT-II cells in the spleen on day 7 p.i. (**D**) Quantification of $PD\text{-}1^{hi}$ OT-II T_{FH} from the plots shown in (B) (**E**) Histogram overlay of Bcl6 expression by $CD6L^-CXCR5^+$ OT-II cells. Relative expression of (**F**) Bcl6 and (**G**) IL-21 mRNA in sorted $CXCR5^+$ OT-II cells relative to naïve $CD4^+$ T cells. Bar graphs in (A), (C), (D), (F) and (G) show mean ± SEM. n=3–5 mice per group, representative of 3–4 independent experiments. *** denotes a p value of <0.001 calculated using a one way ANOVA with Tukey's analysis.

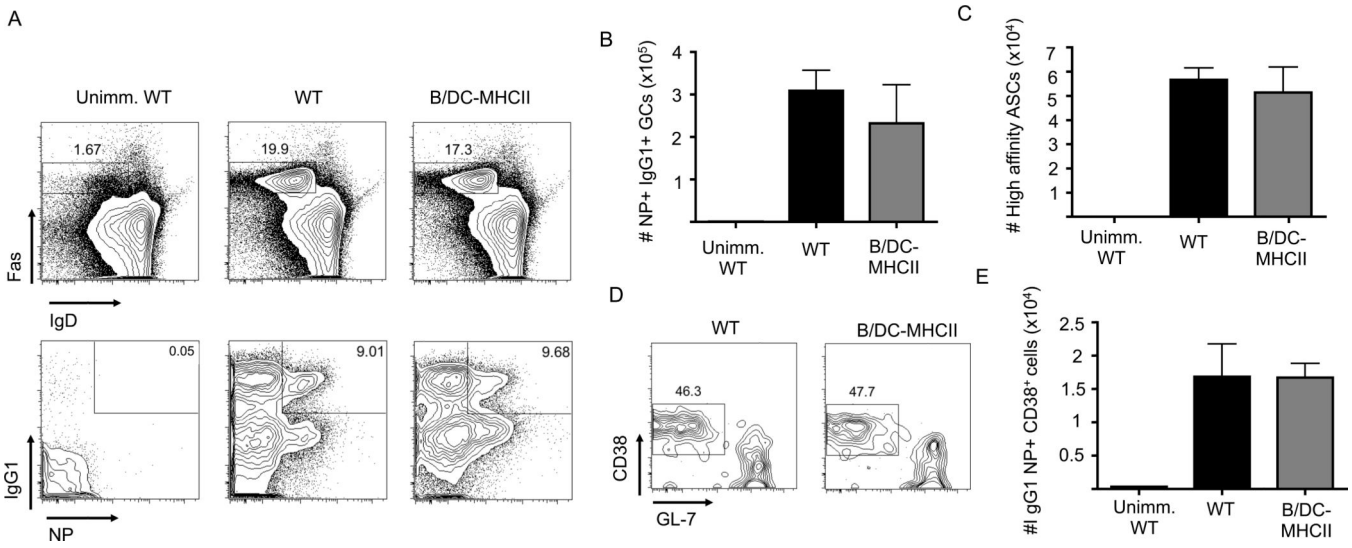

Figure 6.

MHCII dependent antigen presentation by DCs and B cells is sufficient for GC B cell responses after protein immunization. 1×10^5 OT-II cells were transferred to WT and B/DC-MHCII mice and mice were immunized i.p. with NP-OVA in alum. (**A**) Representative FACS plots of splenic Fas^+ GC B cells (gated on $CD19^+$ $B220^+$ cells) (top plots) and IgG1 expression and NP specific cells in the GC population (gated on $CD19^+$ $B220^+$ Fas^+ IgD^{lo} cells) (bottom plots) on d7 p.i. Numbers represent the percentage of B cells that are GCs (top) and percent of GC B cells that are NP^+ $IgG1^+$ (bottom) (**B**) Total number of NP specific $IgG1^+$ GC B cells on d7 p.i. quantified from the plots in (A) (**C**) Total number of high affinity, NP specific $IgG1^+$ ASCs in the spleen on day 14 p.i. as determined by ELISPOT. (**D**) Representative FACS plots of class-switched NP specific memory B cells on d29 p.i. (gated on $CD19^+$ $B220^+$ $dump^-$ $IgG1^+$ IgD^-IgM^- NP^+ cells) (**E**) Number of $IgG1^+$ NP memory B cells on 29 p.i. quantified from the plots in (D). Bar graphs in (B), (C) and (E) show mean ± SEM. n= 4–5 mice per group, representative of 2–3 independent experiments.

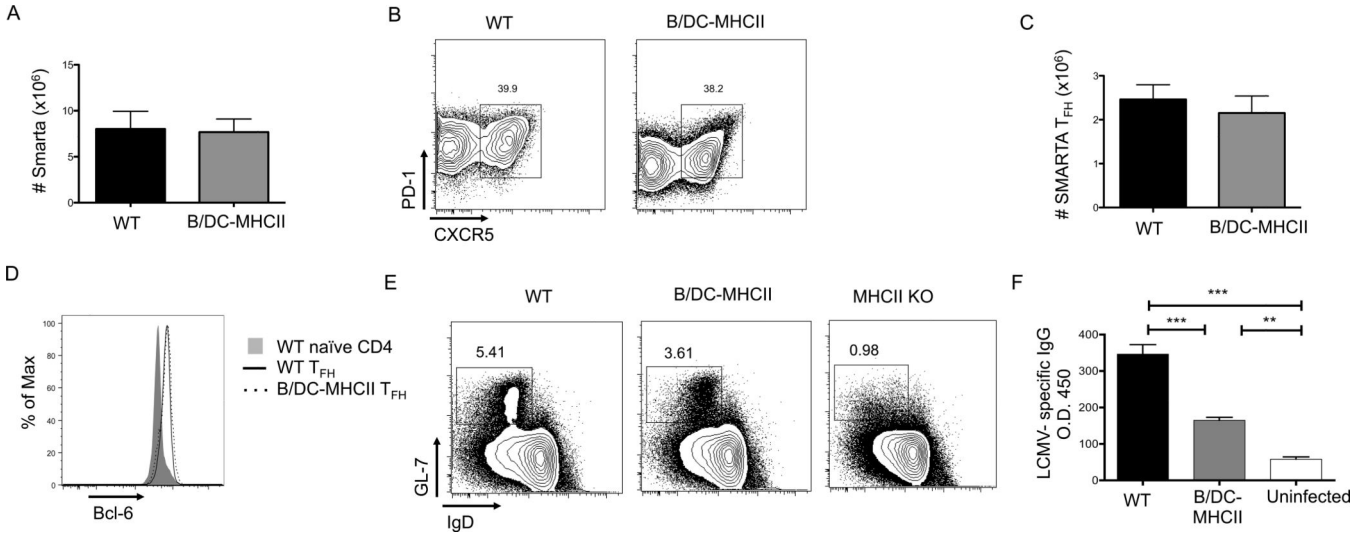

Figure 7.

MHCII antigen presentation by DCs and B cells cooperates for T_{FH} and GC differentiation during LCMV infection. B/DC-MHCII mice received 1×10^7 $CD4^+$ T cells from C57/BL6 mice 7 days prior to infection to reconstitute the $CD4^+$ T cell compartment. 1×10^4 SMARTA transgenic $CD4^+$ T cells were transferred to WT and B/DC-MHCII mice and the mice were infected with LCMV Armstrong one day later. Splenocytes were analyzed on day 8 post infection. (**A**) Total number of SMARTA cells per spleen in WT and B/DC-MHCII mice on d8 post infection (gated on $CD19^-TCR\beta^+$ $CD45.1^+$ cells). Numbers represent the percent of Smarta cells that are $CXCR5^+$ $PD\text{-}1^+$ (**B**) Representative FACS plots of $CD19^-TCR\beta^+$ $CD45.1^+$ Smarta cells for PD-1 and CXCR5 expression. (**C**) Total number of $PD\text{-}1^{hi}$ $CXCR5^+$ Smarta T_{FH} 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 $PD\text{-}1^+$ $CXCR5^+$ Smarta 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 post infection (gated on $CD19^+$ $B220^+$ $F4/80^-$ $GR\text{-}1^-$ $TCR\beta^-$ 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 d8 p.i., compared to uninfected C57/BL6 mice ** denotes a p value of < 0.01 and *** denotes a p value of <0.001 calculated using a one way ANOVA with Tukey's analysis. Bar graphs in (C) and (F) show mean ± SEM. Data are representative of 2 independent experiments with 4–5 mice per group.

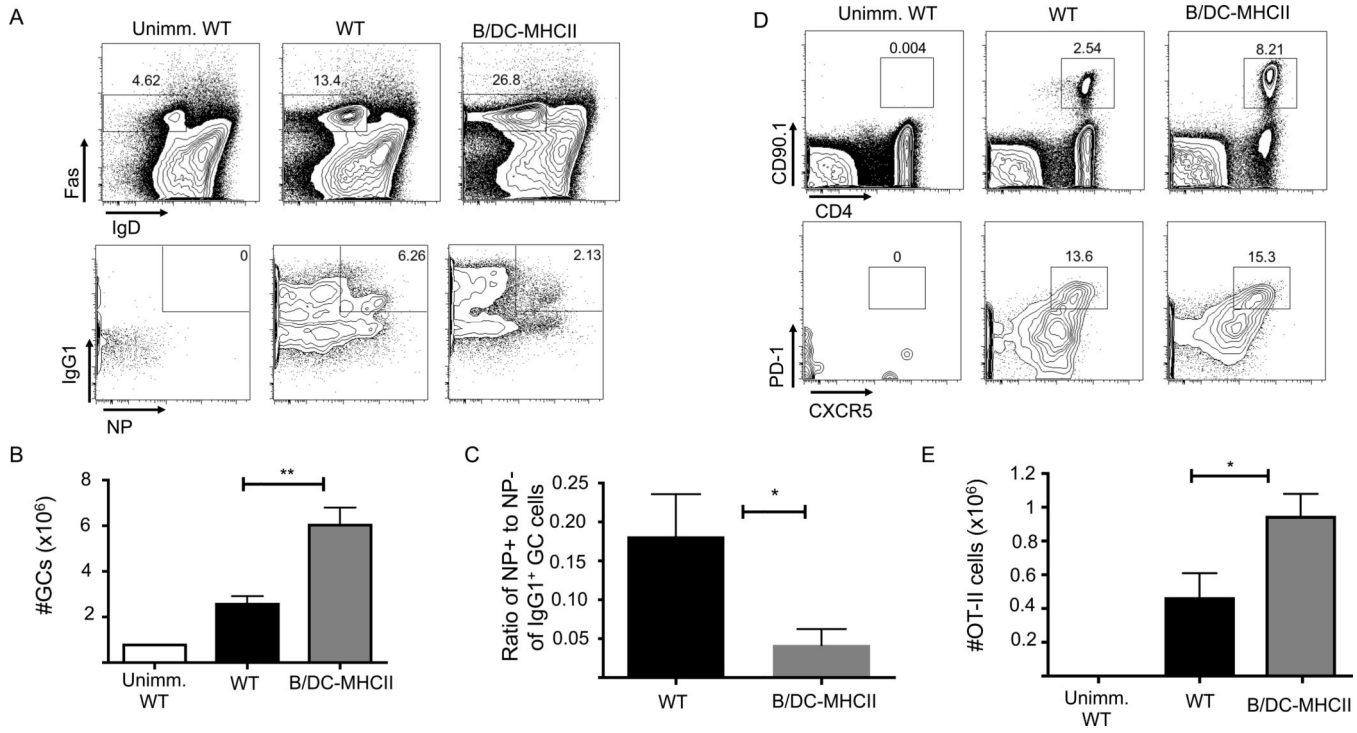

Figure 8.

Increased OT-II and Germinal Center responses in the absence of endogenous $CD4^+$ T cells. 1×10^5 OT-II cells were 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) IgG1 expression and NP specific cells of the GC population (gated on $CD19^+$ $B220^+$ Fas^+ IgD^{lo} cells, bottom plots) on day 14 p.i. Numbers represent the percentage of B cells that are GCs (top) and percent of GC B cells that are NP^+ $IgG1^+$ (bottom). (**B**) Total number of NP specific $IgG1^+$ GCs on day 14 p.i. as quantified from the plots in (A) (**C**) Ratio of the percentage of NP^+ to NP^- cells of $CD19^+$ $B220^+$ IgD^{lo} Fas^+ $IgG1^+$ GC B cells (**D**) Representative FACS plots of $CD19^-$ $TCR\beta^+$ OT-II cells (top) and $CXCR5^+$ $PD\text{-}1^{hi}$ OT-II T_{FH} (bottom) on day 14 p.i. Numbers represent the percent of $CD4^+$ T cells that are OT-II (top) and the percent of OT-II cells that are $CXCR5^+$ $PD\text{-}1^{hi}$ (bottom). (**E**) Total number of splenic $CD19^-$ $TCR\beta^+$ 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 per group, representative of two independent experiments. ** denotes a p value of <0.01 and * denotes a p value of <0.05 calculated with Student's *t* test.

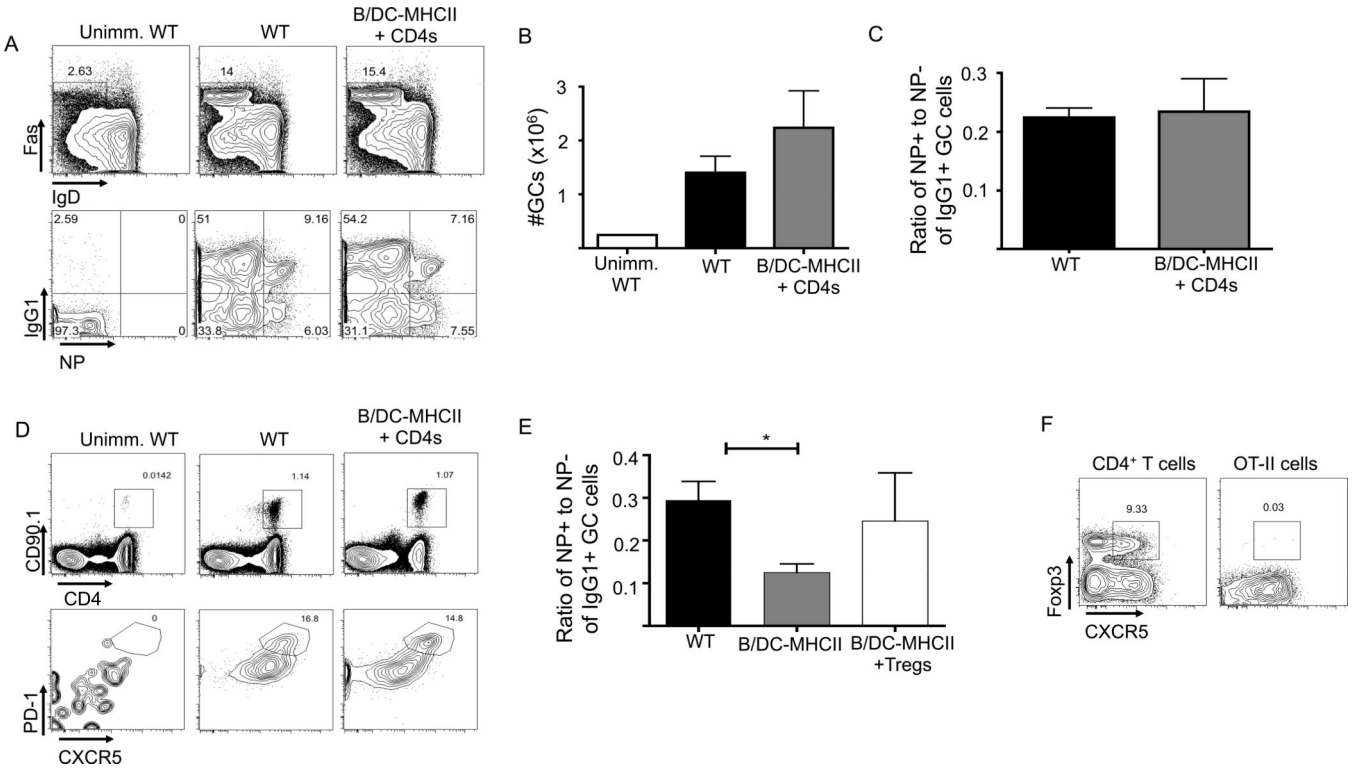

Figure 9.

Germinal Centers and OT-II cell responses in the presence of either polyclonal $CD4^+$ T cells or Tregs. (A–D) 1×10^7 $CD4^+$ T cells from WT mice were transferred to B/DC-MHCII mice. One week later, 1×10^5 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) and NP specific cells in the GC (bottom). Numbers represent the percentage of B cells that are GCs (top) and percent of GC B cells that are NP^+ $IgG1^+$ (bottom). (**B**) Total number of NP specific $IgG1^+$ GCs on d14 p.i. quantified from the plots in (A) (**C**) Ratio of NP^+ to NP^- cells of $CD19^+$ $B220^+$ IgD^{lo} Fas^+ $IgG1^+$ GC B cells (**D**) Representative FACS plots of OT-II cells (top) and OT-II T_{FH} (bottom) on d14 p.i. Numbers represent the percent of $CD4^+$ T cells that are OT-II (top) and the percent of OT-II cells that are $CXCR5^+$ $PD\text{-}1^{hi}$ (bottom). (**E**) 5×10^5 sorted GFP+ Foxp3+ Tregs from FoxP3 GFP mice and 10^5 OT-II cells were 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 $CD19^+$ $B220^+$ IgD^{lo} Fas^+ $IgG1^+$ GC B cells. (**F**) Analysis of Foxp3 and CXCR5 of endogenous $CD4^+$ T cells (gated on $TCR\beta^+$ $CD19^-$ splenocytes) and OT-II cells (gated on $TCR\beta^+$ $CD19^-$ $CD4^+$ $CD090.1^+$) from the spleens of C57/BL6 mice immunized with NP-OVA in alum on day 8 p.i. Numbers represent the percentage of $FoxP3^+$ $CXCR5^+$ cells. * denotes p value of <0.05 using a one way ANOVA with Tukey's analysis. Bar graphs in (B), (C) and (E) show mean ± SEM. n=3–6 mice per group, representative of two experiments. Data in E are pooled from two independent experiments.