IgG-immune complexes promote B cell memory by inducing BAFF[1]

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Abstract

Memory B cell responses are vital for protection against infections, but must also be regulated to prevent autoimmunity. Cognate T cell help, somatic hypermutation, and affinity maturation within germinal centers (GCs) are required for high affinity memory B cell formation; however, the signals that commit GC B cells to the memory pool remain unclear. In this study, we identify a role for IgG immune complexes (ICs), FcγRs, and BAFF during the formation of memory B cells in mice. We found that early secretion of IgG in response to immunization with a T-dependent antigen leads to IC-FcγR interactions that induce DCs to secrete BAFF which acts at or upstream of Bcl-6 in activated B cells. Loss of CD16, hematopoietic cell-derived BAFF, or blocking IC:FcγR regions *in vivo* diminished the expression of Bcl-6, the frequency of GC and memory B cells, and secondary antibody responses. BAFF also contributed to the maintenance and/or expansion of the Tfh population, although it was dispensable for their formation. Thus, early antibody responses contribute to the optimal formation of B cell memory through IgG-ICs and BAFF. Our work defines a new role for FcγRs in GC and memory B cell responses.

Keywords

Rodent; B cells; Dendritic Cells; T Cells; Antibodies; Fc Receptors; Memory; Spleen and Lymph Nodes

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Introduction

Adaptive immunity requires the commitment of activated B cells to either the memory or plasma cell (PC) compartments, the differentiation of CD4$^+$ T cells to follicular helper T cells (Tfh), and coordinated expression of chemoattractant receptors to position T and B cells within the follicle for cognate interactions (1, 2). The specialized microenvironment of the germinal center (GC) provides a site for rapid expansion and selection of B cell clones whose somatically mutating immunoglobulin (Ig) V regions compete for a limiting amount of antigen displayed on follicular dendritic cells and limited availability of T cell help (3–5). Although many steps in the cyclic process of somatic hypermutation and clonal selection are defined, the events that dictate activated B cell fate to GC or to the memory B cell pool are incompletely understood.

During the GC response, Tfh cells are critical effectors that provide help to B cells (6, 7). Tfh cells engage activated B cells at the T:B border, and their secreted cytokines promote Ig isotype switching and the selection of cells with high affinity B cell receptors in GCs (1, 5, 8). The expression of CXCR5, ICOS, PD-1, and the secretion of IL-21 distinguish Tfh cells from other CD4$^+$ T cell subsets (6, 9). The formation of Tfh cells is dependent on the expression of Bcl-6, a process linked to ICOS expression on CD4$^+$ T cells (10) and influenced by IL-2 (11, 12). This commits primed T cells to the Tfh pool and inhibits their differentiation to other T cell subsets (13–16). Bcl-6 is also required for GC B cell formation (17–19). In activated B cells, Bcl-6 downregulates Blimp-1, directing B cells away from PC differentiation and toward the memory pathway (20, 21). Cytokines such as IL-6 and IL-21 have been shown to affect Bcl-6 expression in B and T cells (22–24); however the loss of either cytokine is not enough to eliminate GCs and memory B cells, and a more complete picture of the events upstream of Bcl-6 expression are of interest in understanding B and T cell differentiation in GC responses.

BAFF plays an essential role in controlling the development and survival of B2 and marginal zone B cells (25, 26), enhancing the survival of plasmablasts (27) and affinity-matured B cells in the GC (5). Earlier studies in which BAFF was neutralized or deleted suggest BAFF plays a role in the GC response; however, interpretations of those results are complicated by the global loss of B cells associated with BAFF depletion (28–31). Others have shown that BAFF and anti-CD40 increase ICOSL expression on B cells (32, 33), and that TACI serves to limit the expression of ICOSL and the expansion of Tfh cells and GC B cells (34). Thus, BAFF has been implicated in events that contribute to GC responses; however, how BAFF is induced and where it acts in the GC response remains unclear.

In this study we identify a previously unrecognized role for IgG-ICs, CD16 (FcγRIII), and BAFF in the formation of B cell memory. We found that early production of anti-NP-IgG promotes the formation of ICs that activate DCs through CD16. This induces the secretion of BAFF, which acts at or upstream of Bcl-6 to promote the formation of GC B cells and proper memory cell formation. Although BAFF is not involved in the formation of Tfh cells, it plays a role in stabilizing and/or expanding the population at the peak of the GC response. Thus, IgG-ICs and CD16, through BAFF, act at or upstream of Bcl-6 expression in GC B

cells and in the maintenance and/or expansion of Tfh cells to support the optimal formation of B cell memory during NP-specific immune responses.

Materials and Methods

Animals

B6-Ly5.2 congenic mice were purchased from NCI, BAFF$^{-/-}$ mice (29), and CD16-2$^{-/-}$ (FcγRIV) mice (35) were obtained from, Glenn Matsushima, and Charles Jennette at UNC-Chapel Hill. CD16$^{-/-}$ and CD64$^{-/-}$ mice (36) were obtained from Dr. Anne Sperling at the University of Chicago, and tissue from BAFF Tg mice (37) from Jeffrey Rathmell at Duke University (MMRRC strain #36508, B6.Cg-Tg(CD68-Tnfrsf13c)MB21Nemz/Mmucd). CD32$^{-/-}$ mice (38) were purchased from Jackson labs. Mice were used at 8–12 weeks of age and maintained in an accredited animal facility.

Reagents and Antibodies

Antibodies against mouse CD4, CD19, CD95, GL-7, ICOS, ICOSL, PD-1, and B220-647 were purchased from Biolegend, CXCR5, B220, IgG$_1$, IgG$_{2a}$, IgG$_{2b}$, and IgG$_3$ antibodies from BD Biosciences, Bcl-6, XBP-1, and IRF-4 from Santa Cruz, and BAFF (1C9) from Enzo. Anti-μ F(ab)$_2$ was purchased from Jackson ImmunoResearch. Anti-μ (clone B7.6), anti-NP (clones H33L and B1-8), 2.4G2 (FcγRIIb /FcγRIII block) and Ac38 idiotype antibodies were purified from hybridoma supernatants. (Ac38 is an idiotype antibody that recognizes B-1-8 specificities generated during NP immunization). Recombinant mouse BR3-Fc and isotype control protein were generated using mammalian expression systems and standard purification protocols. H33L and B1-8 were gifts from Dr. Garnett Kelsoe (Duke University). IL-4 and IL-5 were purchased from Peprotech, recombinant BAFF (rBAFF) from R&D Systems, NP-OSu from Biosearch Technologies, KLH and PNA-biotin from Sigma Aldrich, alum from Thermo Scientific, and streptavidin-Alexa 488 and Alexa 647 from Invitrogen. Streptavidin-alkaline phosphatase and anti-IgG alkaline phosphatase were purchased from Southern Biotech. The Fc-binding TG19320 peptide was synthesized as described (39, 40).

B cell purification and bone marrow derivation of MFs and DCs

Splenic B cells were isolated from B6 mice by negative selection (StemCell Technologies) and were 95–99% pure, as determined by flow cytometry. Splenic DCs were purified by positive selection of CD11c$^+$ cells (Miltenyi) from enriched low-density cells (OptiPrep; Sigma). Purified cells were 80% CD11c$^+$.

Bone marrow-derived DCs (BMDCs) and bone marrow-derived MFs (BMMFs) were prepared from single cell suspensions from the tibias and femurs of B6, CD64$^{-/-}$, CD32$^{-/-}$, CD16$^{-/-}$, and BAFF$^{-/-}$mice. Following RBC lysis, cells were cultured 7 days in a 24 well low-cluster plate (Costar 3471) with 10 ng/ml GM-CSF and IL-4 to derive DCs and in 20 ng/ml M-CSF to derive MFs.

Cell Culture

We pre-formed immune complexes by stimulating B6 B cells (1.5×10^5) with an excess of anti-μ (B7.6; IgG_1; 30 μg/ml). The polyclonal IgM produced after seven days forms a complex with the excess anti-μ resulting in IgG1-IgM ICs in the supernatant. These B cell supernatants were used as a source of ICs in preparing DC conditioned medium (DC CM).

DC CM was prepared by culturing 2×10^4 BMDCs (day 7) in a 96 well plate in the presence of IC-containing supernatants (see above; 20% of volume), IL-4 (25 ng/ml), and IL-5 (25 ng/ml). After seven days, supernatants were harvested and frozen at −80°C.

For *in vitro* co-cultures, 1.5×10^5 purified B6 B cells were co-cultured with 1×10^4 BMDCs or *ex vivo* DCs in a 96 well plate stimulated with IL-4 (25 ng/ml), IL-5 (25 ng/ml) and 30 μg/ml anti-μ with or without recombinant murine BAFF (5 ng/ml) or DC CM (20% of total volume). Intracellular Bcl-6 was assessed by flow cytometry after 48 hours.

ELISAs

NP-specific IgG levels were quantitated from serum using microtiter plates coated with $NP_{13}BSA$ and blocked with 0.5% BSA. Serially diluted serum samples were incubated overnight at 4°C. Anti-NP was detected using an alkaline phosphatase conjugated rabbit anti-mouse IgG antibody (1/1000 dilution) followed by phosphatase substrate. Optical density (OD) values were converted to concentration based on standard curves using the H33L (anti-NP) hybridoma.

ELISpot

For the analysis of NP-specific B cells, multiscreen ELISpot plates (Millipore) were coated with $NP_{13}BSA$ in PBS and blocked with 1% BSA. Single cell suspensions of spleen were prepared from immunized or naïve B6 mice. After RBC lysis, cells were plated in serial dilutions on washed ELISpot plates. Anti-NP IgG-secreting spots were detected with anti-IgG-biotin and streptavidin-HRP (BD Biosciences). Plates were developed with 3-amino 9-ethylcarbazole.

To enumerate BAFF-secreting DCs, $CD11c^+$ cells (1×10^6) were purified from spleens and cultured for 60 hours on BR3-Fc coated ELISpot plates. BAFF-secreting cells were detected using anti-BAFF (clone 1C9). To enumerate BAFF secreting cells from BMDCs, day 7 cells (2.5×10^5) were plated on ELISpot plates as above and incubated 24 hours with preformed ICs (IgM + anti-μ or NP-OVA + anti-NP IgG monoclonal Ab, H33L) prior to addition of 1C9. Anti-μ ICs were made by combining the supernatant from stimulated B cells (20 ng of IgM) with anti-μ (5 μg) or by combining anti-NP IgG with NP-OVA. In some experiments, TG19320 was added at 50 μg/ml to inhibit IgG binding to FcγRs.

Bone Marrow Chimeras

B6-Ly5.2 congenic mice (6–8 weeks of age) were lethally irradiated (10.5 Gy; 1050 rads) and reconstituted with 8×10^6 bone marrow cells from either B6 (B6 control chimeras) or $BAFF^{-/-}$ ($BAFF^{-/-}$ chimeras) mice. After 8 weeks, we monitored reconstitution by assessing the frequency of $CD45.1^+$ and $CD45.2^+$ splenocytes by flow cytometry.

Immunization and Adoptive Transfers of BMMF/BMDCs

B6, BAFF$^{-/-}$ bone marrow chimeras, and CD16$^{-/-}$ mice (8–10 weeks of age) were immunized by i.p. or s.c. injection with 100 μg of NP$_{14}$KLH precipitated in an equal volume of alum (Imject® Thermoscientific). Mice were boosted by i.v. injection with the same dose of soluble NP$_{14}$KLH at day 35. To assess the contribution of DCs or MFs in the secretion of BAFF, 8×10^6 BAFF Tg or BAFF$^{-/-}$ BMDCs or BMMFs were injected at the time of s.c. immunization. Draining lymph nodes were harvested on day 7 for flow cytometry analysis.

TG peptide injections

B6 mice were immunized with 100 μg NP$_{14}$KLH in alum (1:1) via i.p. injection and administered three (i.p.) injections (15–30 mg/kg) of Fc blocking peptide (TG19320) or equal amount of unrelated control peptide over the course of seven days.

Flow Cytometry

GC B cells and Tfh were analyzed on day 7 post-immunization and were defined as CD19$^+$, GL-7$^+$, CD95$^+$ and CD4$^+$, CXCR5$^+$, PD-1$^+$. Ac38 was used to define NP-specific GC B cells. NP-specific memory B cells were defined as Ac38$^+$ IgG$^+$ double positive CD19+ lymphocytes. The lymphocyte gate was determined by forward and side scatter properties. To gate on Tfh populations, initially used isotype control antibody staining for CXCR5. To gate on GC B cells, we used fluorescence minus one CD95 (CD19 PB + GL7 FITC+) and for GL7 (CD19 PB + CD95 PE+). All subsequent gating was based on untreated B6 mice. To quantitate expression of intracellular IRF-4, Bcl-6 and XBP-1, splenocytes from immunized B6, CD16$^{-/-}$, B6 control chimeras and BAFF$^{-/-}$ chimeras mice were washed, fixed (4% paraformaldehyde), and permeabilized with methanol for a minimum of 24 hours at −20°C. Fixed cells were washed and blocked with 2.4G2 before staining. Data are expressed as fold change in MFI/isotype control MFI.

Real Time PCR

Splenic B cells from B6 and BAFF$^{-/-}$ chimeras were purified after NP$_{14}$KLH immunization. mRNA was isolated from 5–10×10^6 purified B cells and cDNA synthesized using Superscript VILO cDNA Synthesis Kit (Invitrogen). DNA was subsequently amplified using FastStart Universal SYBR Green Master mix (Roche). Relative values were compared using the $2^{-\Delta\Delta CT}$ method. 18s rRNA was used as an internal control in all experiments. Primers included: murine *Aicda* forward 5'GGGAAAGTGGCATTCACCTA3', murine *Aicda* reverse 5'GAACCCAATTCTGGCTGTGT3' murine 18s rRNA forward 5'TCAAGAACGAAAGTCGGAGGTT3', murine 18s rRNA revese 5'-GGACATCTAAGGGCATCACAG-3'.

Germinal Center Staining and Counting

Spleens were harvested from B6 or CD16$^{-/-}$ mice on days 7, 14, and 21 after immunization and flash frozen in OCT (Optimum Cutting Temperature; Fisher). Tissue sections (6 micron) were fixed in 1:1 MeOH/Acetone, blocked with 10% FBS in PBS containing 2.4G2, and stained with PNA-biotin and B220-Alexa647, and Streptavidin-Alexa488. Germinal centers were defined as PNA$^+$ cell clusters within B220$^+$ follicles (41). The number of germinal

centers per mm^2 of $B220^+$ area was determined by dividing the number of germinal centers counted in a field by the area of $B220^+$ follicles in the same field. This accounted for follicles that were only partially represented in a given field (42). This was done for 10–30 fields per mouse, totaling 30–100 follicles per mouse at each time point.

Microscopy

Macroscope images were obtained on a Leica MX16FA fluorescence stereo microscope/ macroscope (0.63× objective; numerical aperature of 1.0). Other images were obtained using an Olympus Fluoview 500 (10× objective; numerical aperature of 0.45).

Statistics

The one-sided, or one-sample *t* test was used to compare changes in transcription factor levels, and ELISpot. Two-sample student *t* test was used to assess statistical differences between cell populations measured by flow cytometry, and serum antibody secretion. Analyses were performed in GraphPad Prism.

Results

BAFF$^{-/-}$ bone marrow chimeras exhibit reduced secondary responses

Previous studies have linked BAFF with an enhanced response to vaccination, suggesting that it plays a role in memory responses (43–45). To assess this, we generated BAFF$^{-/-}$ bone marrow chimeras by engrafting irradiated B6 mice with B6 (BAFF$^{+/+}$) or BAFF$^{-/-}$ bone marrow. This approach limits BAFF deficiency to hematopoietic cells, allowing other sources of BAFF to maintain the peripheral B cell population (46). No differences in the spleen cellularity between B6 and BAFF$^{-/-}$ bone marrow chimeras were observed (Supplemental Figure 1A), and the basal level (d0) of Tfh and GC B cells were not different. In BAFF$^{-/-}$ chimeras, we found that the primary IgG response to $NP_{14}KLH$ (Figure 1A) was comparable to B6 control chimeras. However, 7 days after secondary immunization (day 42), BAFF$^{-/-}$ bone marrow chimeras showed a 1.4-fold reduction in the levels of IgG, and 14 days after secondary immunization (day 49) the levels of IgG were reduced 2-fold compared to B6 chimeras (Figure 1B). Diminished production of IgG during the secondary response could reflect diminished class switch since BAFF can induce AID expression (47, 48). However, AID mRNA levels in B cells from B6 control and BAFF$^{-/-}$ chimeras were not different (Supplemental Figure 1B), suggesting that BAFF has a role other than in class switch.

BAFF$^{-/-}$ chimeras exhibit defects in the frequency of memory B, Tfh cells, and GC B cells

Rapid, high titer secondary immune responses require the activation of memory B cells (49). Although most IgG memory B cells do not require BAFF for maintenance (50), it is not known whether BAFF is important for their formation. To determine whether BAFF affects the frequency of memory B cells, we immunized (i.p.) BAFF$^{-/-}$ chimeras with $NP_{14}KLH$ and measured the frequency of NP-specific memory B cells (CD19$^+$IgG$^+$Ac38 Id$^+$) on day 28 post-immunization. We found that immunization significantly increased the frequency of memory B cells in B6 and BAFF$^{-/-}$ bone marrow chimeras; however, the magnitude of the response was significantly lower in BAFF$^{-/-}$ chimeras (Figure 1C).

Tfh cells are critical in the early GC response and required for the differentiation of memory B cells (51, 52). It was possible that BAFF affected memory responses by influencing Tfh cells that in turn affected GC responses. To assess whether BAFF affects formation and/or maintenance of Tfh cells, B6 and BAFF$^{-/-}$ chimeras were immunized and the frequencies and numbers of Tfh cells (CXCR5$^+$PD-1$^+$CD4$^+$) were quantitated on days 3 and 7 post-immunization (Figure 1D, 1E, and Supplemental Figure 1C). On day 3, the frequency of Tfh cells in B6 chimeras increased by 1.5-fold while in BAFF$^{-/-}$ chimeras it increased 1.2-fold. This suggests that BAFF does not play a significant role in the formation of Tfh cells. However on day 7 post-immunization, the frequency and number of Tfh cells in B6 chimeras increased an additional 2 fold, whereas their frequency in BAFF$^{-/-}$ chimeras did not change. This suggests that BAFF may support the expansion of Tfh cells after pre-Tfh cells transition to Tfh.

Germinal centers are necessary for the formation of high affinity, class-switched memory B cells (53, 54). To determine whether BAFF impacted the frequency of GC B cells, we enumerated CD19$^+$GL-7$^+$CD95$^+$ GC B cells 7 days after immunization. In BAFF$^{-/-}$ chimeras, the frequency and number of total GC B cells (Figure 1F, 1G, and Supplemental Figure 1D), and the frequencies of Ag-specific (Figure 1H; CD19$^+$Ac38$^+$ GL-7$^+$CD95$^+$) GC B cells in BAFF$^{-/-}$ chimeras were significantly lower than those in B6 chimeras. Thus, BAFF significantly contributes to optimal antigen-specific GC responses.

BAFF acts at, or upstream of, Bcl-6 expression in B cells

Bcl-6 plays a critical role in initiating GC responses and committing activated B cells to a memory cell phenotype (17, 19). Thus, one possibility was that the loss of BAFF negatively affected Bcl-6 expression. To test this, we measured Bcl-6 levels in GC B cells after immunization. We found that on day 7, the levels of Bcl-6 in GC B cells from immunized BAFF$^{-/-}$ chimeras were decreased 40% compared to B6 chimeras (Figure 1I and 1J). The data show that hematopoietic cell-derived BAFF acts at, or upstream of, Bcl-6 expression in B cells. Collectively, our data indicate that BAFF impacts the formation of GC and memory B cells by increasing the expression of Bcl-6 in GC B cells, and indirectly through maintaining the Tfh cell populations during the GC response.

DC-derived BAFF regulates the frequency of GC B and Tfh cells

The BAFF$^{-/-}$ chimera model is characterized by the absence of BAFF in all hematopoietic cells. Among bone marrow-derived cell types that are capable of producing BAFF, myeloid cells are a major source of BAFF following infection or immunization (27, 43, 55). To determine whether myeloid cells are a sufficient source of BAFF during GC B cell and memory B cell fate decisions, we adoptively transferred bone marrow-derived dendritic cells (BMDCs) and bone marrow-derived macrophages (BMMFs) from BAFF transgenic (Tg) mice into BAFF$^{-/-}$ chimeras by s.c. injection at the time of immunization. We previously established that 70% of s.c. injected BMDCs migrated to the inguinal lymph nodes, and that the magnitude of the s.c. anti-NP response was comparable to i.p. immunization (data not shown). We found that constitutive expression of BAFF by Tg DCs, but not Tg MFs, restored the frequencies of GC B cells (Figure 2A), Tfh cells (Figure 2B), and the levels of Bcl-6 in GC B cells (Figure 2C) in BAFF$^{-/-}$ chimeras. Since these BAFF Tg MFs secrete

more BAFF, than BAFF Tg DCs (37), the responses were not due to higher production of BAFF by Tg DCs. Conversely, transfer of BAFF$^{-/-}$ DCs did not increase the frequencies of GC B cells (Figure 2A) indicating that the effects of the transfer on GC B cells were not due to an increased number of DCs, or to secretion of other cytokines made by the transferred DCs. This suggests that in addition to DCs presenting antigen during GC responses (56) DC-derived BAFF is also be important in directing the differentiation of GC cell populations.

The binding of immune complex to FcγRs induces BAFF secretion

Previous studies showed that exogenous ICs induce BMDCs to secrete a number of cytokines, including BAFF (57, 58). In another study, mice lacking the common gamma chain of the FcγRs (Fcγ$_c$) exhibited diminished secondary immune responses (59). Because our data suggest that DCs may promote secondary responses via BAFF, we postulated that ICs formed by the early IgG antibody response might induce DCs to secrete BAFF. This model requires that the early IgG response occur concurrently with, or precede BAFF secretion. To test this, we harvested spleens from B6 mice on days 2, 3, and 7 following NP$_{14}$KLH immunization and used ELISpot to measure the numbers of antibody (IgG) secreting cells (ASCs) and BAFF-secreting DCs. NP-specific (IgG) ASCs were increased 7-fold by day 2 post-immunization and expanded to 90-fold over the course of 7 days (Figure 2D). Similarly, the number of splenic CD11c$^+$ DCs that secreted BAFF increased 6-fold between days 2 and 7 (Figure 2E). Thus, secretion of Ig by B cells and production of BAFF by DCs occur concomitantly, beginning approximately 2 days following immunization. This supports the idea that IgG-ICs formed early in immune response contribute to the production of BAFF, which is required to optimize GC responses.

To further test the idea that IgG-ICs induce DCs to secrete BAFF, we blocked IgG-Fc:FcγRs interactions *in vitro* and assessed whether this impacted BAFF secretion by DCs. Stimulation of B6 BMDCs with pre-formed IgG-ICs (IgM bound by anti-μ of IgG1 isotype) induced a 1.8-fold increase in BAFF secretion (Figure 2F). This was not unique to IgM/IgG ICs because preformed anti-NP ICs (NP-OVA bound by anti-NP of IgG1 isotype) induced a dose dependent 2.5-fold increase in BAFF secretion (Supplemental Figure 1E). Co-culture with a tetrameric tripeptide (TG19320) that blocks IgG Fc regions (39, 40) reduced the number of BAFF-secreting DCs to levels indistinguishable from unstimulated cells. To assess whether blocking Fc:FcγR interactions affected the adaptive immune response *in vivo*, we administered TG19320 to B6 mice at the time of immunization and measured the frequencies of GC B and Tfh cells. We found that co-administration of TG19320 with NP$_{14}$KLH blocked an increase in GC B and Tfh cells on day 7 (Figure 2G and 2H). This indicates that the interactions between IgG-ICs and FcγRs are necessary for optimal GC responses and for the maintenance and/or expansion of newly formed Tfh cells in response to immunization. Collectively, these data identify a mechanism wherein IgG-ICs formed early in the immune response ligate FcγRs on DCs to induce BAFF secretion, which in turn contributes to optimal GC responses.

BAFF regulates the expression of Bcl-6 in activated B cells *in vitro*

To define whether BAFF plays a role in committing activated B cells to the memory compartment, we established an *in vitro* reconstitution system using the expression of Bcl-6

as a marker of memory B cell commitment. In this *in vitro* system, we used conditioned medium prepared from B6 BMDCs (DC CM) stimulated with preformed IgG1-ICs (Figure 2F) as a source of BAFF. This was based on in *vivo* findings that GC responses were dependent of BAFF produced by DCs (Figure 2A–C). To generate activated B cells, we used purified splenic B cells (B6) stimulated with anti-μ in combination with IL-4 and IL-5 to induce a low level of Bcl-6 expression (Figure 3A/B; B cell activation). Thus, changes in Bcl-6 as a consequence of DC CM or recombinant BAFF could be measured. We found that DC CM increased Bcl-6 expression in activated B cells approximately 2-fold compared to cells cultured in the absence DC CM (Figure 3A and 3B). These levels were comparable to those achieved with recombinant BAFF. Further, CM from BMDCs stimulated with F(ab')$_2$-containing ICs was not as efficient as CM stimulated with IgG-ICs (intact Fc regions) at inducing Bcl-6. In this *in vitro* system, Bcl-6 expression was not elevated in B cells cultured with DC CM where BAFF was neutralized with BR3-Fc, or where DC CM was made from BAFF$^{-/-}$ BMDCs (Figure 3C). These data showed that DC-derived BAFF, induced by IgG-ICs, directly affects Bcl-6 expression in B cells activated *in vitro*.

As Bcl-6 levels become elevated, the PC program is attenuated (60, 61). To further validate the *in vitro* model, we measured intracellular IRF-4 and XBP-1 and found that DC CM diminished the levels of both transcription factors by 2.2-fold (Figure 3D–G), indicating that BAFF acts at or upstream of Bcl-6, directing B cell differentiation away from a PC fate. To test whether BAFF reduces PC differentiation *in vivo*, we enumerated splenic PCs from immunized B6 chimeras and BAFF$^{-/-}$ chimeras at day 5 (Figure 3H), a time when PCs normally appear in the spleen (62). We found that CD138+B220– cell numbers in BAFF$^{-/-}$ chimeras were increased 2.7-fold compared to B6 chimeras. This indicates that *in vivo*, BAFF decreases PC differentiation as the memory response initiated.

IgG-ICs bind CD16 during the anti-NP response

We reasoned that if FcγR stimulation promoted BAFF secretion, loss of the FcγR that binds IgG1-anti-NP ICs would negatively affect the ability of DC CM to promote Bcl-6. To assess this, we tested whether CMs from BMDCs derived from B6 mice, or mice deficient in CD64 (FcγRI$^{-/-}$), CD32 (FcγRIIb$^{-/-}$), CD16 (FcγRIII$^{-/-}$), or CD16-2 (FcγRIV$^{-/-}$) induced Bcl-6 in the *in vitro* reconstitution system described above. DC CM from CD16$^{-/-}$ mice failed to induce Bcl-6 expression, while CMs from all other FcγR deficient mice induced Bcl-6 to levels comparable to, or above those induced by B6 DC CM (Figure 3I). This suggests that ligation of CD16 on DCs is required for the expression of Bcl-6 in activated B cells during the anti-NP response.

Impaired secondary responses in CD16$^{-/-}$ mice

Our *in vitro* data indicate that CD16 is responsible for inducing DCs to secrete BAFF after NP$_{14}$KLH immunization. To test this *in vivo*, we quantitated the number of splenic BAFF secreting DCs in B6 and CD16$^{-/-}$ mice 7 days after immunization (Figure 4A). In the absence of CD16, we found that the number of BAFF secreting DCs was markedly diminished compared to wild type mice. This is consistent with the idea that CD16 is the FcγR responsible for initiating the IgG1-dominant immune response to NP$_{14}$KLH (54). It is

likely that other Fc receptors would be utilized during immune responses that generate antibodies of IgG subclasses other than IgG1.

To test whether loss of CD16 *in vivo* impaired adaptive immune responses, we measured primary and secondary antibody responses in B6 and CD16$^{-/-}$ mice following NP$_{14}$KLH immunization. As in the BAFF$^{-/-}$ chimeric mice, the primary IgG response in CD16$^{-/-}$ mice was comparable to B6 mice (Figure 4B). However, in the secondary response, the levels of IgG in B6 mice increased 1.7-fold on day 42 (7 days after boost), and 4.5-fold on day 49 (14 days after boost), while IgG levels in the CD16$^{-/-}$ mice did not increase on days 42 or 49 (Figure 4C). This was not an indirect consequence of altered spleen cellularity or changes in the splenic cell populations due to CD16 deficiency since the frequencies of DCs, T, and B cells in CD16$^{-/-}$ mice were not different than B6 mice, and the number of splenocytes from B6 and CD16$^{-/-}$ mice were comparable (Supplemental Figure 1F). This indicates that CD16 plays a role in generating memory B cells and secondary immune responses to NP$_{14}$KLH.

CD16$^{-/-}$ mice exhibit defects in forming GC and memory B cells and maintaining Tfh cells

The loss of DC-derived BAFF in CD16$^{-/-}$ mice supports a role for CD16 in the memory response to NP$_{14}$KLH. To assess whether the diminished secondary response in CD16$^{-/-}$ mice reflects a reduction in memory B cells, we assessed the frequency of NP-specific memory B cells (CD19^{+}Ac38^{+}IgG^{+}) in B6 and CD16$^{-/-}$ mice 28 days following immunization. CD16$^{-/-}$ mice showed a 2.4-fold decrease in the Ac38 Id^{+} IgG memory B cell population compared to immunized B6 mice (Figure 4D and 4E), indicating that CD16 regulates memory responses in part through BAFF production.

Data from immunized BAFF$^{-/-}$ chimeras suggests that BAFF contributes to B cell memory by affecting the GC B and Tfh cell populations. If the binding of IgG-ICs to CD16 were the predominate source of BAFF, then loss of CD16 would also diminish the GC B and Tfh pools. To assess this possibility, we quantitated Tfh cells (CD4^{+}CXCR5^{+}PD-1^{+}) from immunized B6 and CD16$^{-/-}$ mice. After 3 days, CD16$^{-/-}$ mice had a comparable frequency of Tfh cells compared to immunized B6 mice (Figure 4F). However on day 7, CD16$^{-/-}$ mice had 3-fold fewer Tfh cells, similar to the defect in maintaining Tfh cells observed in immunized BAFF$^{-/-}$ chimeras (Figure 1E). After 10 days, frequencies of Tfh cells decreased 2-fold in B6 mice, while the frequencies in CD16$^{-/-}$ mice were not changed compared to day 7. This suggests that BAFF plays a role in the expansion and/or maintenance of Tfh cells during early GC responses, but that other factors also contribute. Adoptive transfer of BAFF transgenic, but not BAFF$^{-/-}$ BMDCs into immunized CD16$^{-/-}$ mice, restored the frequency of Tfh cells on day 7 to levels seen in B6 mice (Figure 4G), suggesting that the defect in CD16$^{-/-}$ mice was a consequence of reduced BAFF. These data demonstrate that CD16 ligation by IgG-ICs induces DCs to secrete BAFF and that CD16 is necessary for memory responses. Though our findings collectively show that binding of IgG-ICs to CD16 contributes to B cell memory through the effects of BAFF on Bcl-6 expression in GC B cells, and maintaining and/or expanding Tfh cells, sources of BAFF other than CD16, or factors other than BAFF may also play a role since loss of either BAFF or CD16 did not completely ablate these populations.

Loss of CD16 diminishes germinal center responses

To assess whether loss of CD16 diminished GCs, we measured the frequency of splenic GC B cells (CD19$^+$GL-7$^+$CD95$^+$) and Ac38 Id$^+$GC B cells 7 days after immunization. We found that both populations of GC B cells were significantly reduced in CD16$^{-/-}$ mice (Figure 5A–C). Consistent with the diminished frequency of GC B cells, we found that the GCs in CD16$^{-/-}$ mice were 54% smaller on day 7 post-immunization, but as the size of the B6 GCs declined (by day 14 and 21) the sizes became similar (Figure 5D and Supplemental Figure 2). The smaller size of the CD16$^{-/-}$ GC on day 7 was not the result of delayed kinetics because by day 28, the GCs in both B6 and CD16$^{-/-}$ mice were diminished, suggesting a comparable duration of the response (data not shown). We also found that immunized CD16$^{-/-}$ mice displayed a 2.3-fold reduction in the number of GCs on day 7; however, the differences were less apparent on day 14. By day 21, the CD16$^{-/-}$ mice had a similar number of GC structures (Figure 5E). Thus, although GCs form in the absence of CD16, they are reduced in number and size early during the immune response. This suggests that stimulation of CD16 is an early event that enhances the GC response and subsequently optimizes the formation of memory B cells.

CD16 and BAFF are required for the expression of Bcl-6 and the formation of GC and memory B cells

Our data indicate that CD16 and IgG-ICs are required for DCs to make BAFF in response to NP$_{14}$KLH (Figure 4A). To address whether DC-derived BAFF was sufficient to restore the GC and memory B cell pools in the CD16$^{-/-}$ mice, we adoptively transferred BMDCs from BAFF Tg mice at the time of immunization. We found that BAFF Tg DCs restored the frequency of GC B cells on day 7 (Figure 6A), and the frequency of Ac38 Id$^+$ memory B cells on day 28 (Figure 6B). This suggests that the lack of B cell memory responses in CD16$^{-/-}$ mice was due to lack of BAFF, and not to intrinsic defects in B cells. In contrast, BMDCs derived from BAFF$^{-/-}$ chimeras were unable to restore GC or memory B cells (Figure 6A and 6B). These results also emphasize that the defects observed in CD16 deficient mice are mediated by BAFF, since DCs from BAFF$^{-/-}$ chimeras have intact CD16.

Our data suggest that DC-derived BAFF acts at or upstream of Bcl-6 (Figure 1I–J, 2C, 3A–C) and downstream of CD16 (Figure 4A). Thus, the absence of CD16 should also lead to diminished Bcl-6 expression after NP immunization, and restoring DC-derived BAFF by BMDC transfer should restore Bcl-6 levels. To test this, we adoptively transferred BAFF Tg or BAFF$^{-/-}$ BMDCs into CD16$^{-/-}$mice at the time of immunization. We found that 7 days after immunization, the expression levels of Bcl-6 in CD16$^{-/-}$ GC B cells were 60% lower compared to B6 controls. Transfer of BAFF Tg BMDCs, but not BAFF$^{-/-}$ BMDCs, restored Bcl-6 levels in CD16$^{-/-}$ GC B cells (Figure 6C). Thus, loss of CD16 reduces BAFF, which impacts Bcl-6 expression in GC B cells. Collectively, the data show that IgG-ICs induce DC-derived BAFF through ligation of CD16. BAFF promotes optimal B cell memory responses by inducing Bcl-6 expression in GC B cells and by maintaining and/or expanding Tfh cells.

Discussion

Interactions between T cells, B cells, and DCs are necessary for the proper execution of the adaptive immune response. This study identifies a previously unappreciated role for FcγRs, and BAFF in the early events of the GC response. We show that ICs formed during the early IgG response to $NP_{14}KLH$ induced the production of BAFF by DCs. BAFF acted at, or upstream of Bcl-6 to promote the optimal formation of GC B cells and to maintain and/or expand newly formed (day 7) Tfh cells. This series of events depends on the formation of IgG-ICs, suggesting that productive early antibody responses contribute to the optimal formation of B cell memory. This mechanism may ensure that an antigen-specific antibody response has occurred prior to initiating the events that promote B cell memory.

In the absence of CD16, the diminished formation of GC and memory B cells, and reduced expansion of Tfh cells (Figures 4–6), coupled with observations that mice deficient in the Fc common gamma chain ($Fc\gamma_c$) exhibit diminished secondary antibody responses (59) imply that IgG is required for optimal adaptive immune responses. We detected antigen-specific IgG by ELISpot within 2–3 days of immunization. The source of this IgG could be extrafollicular PCs because these cells class switch (63) and produce significant local levels of IgG early in the immune response (64, 65). By days 14 and 21, the sizes and numbers of GCs were similar in B6 and CD16-deficient mice, suggesting that this mechanism is involved in pre- or early GC responses. This may also reflect a reduction in available antigen, which would reduce IC formation and make any differences mediated by IC:FcγR interactions less apparent. Consistent with a role for early GC events in the formation of B cells memory, we also found that in the absence of CD16, the average GC size and the number of GCs were diminished on day 7. Further, others showed that in the absence of soluble IgG, the kinetics of secondary GCs were disrupted (66). Thus, IgG plays a role in both primary and secondary GC responses.

Temporally, anti-NP IgG production by ASCs was coincident with BAFF production by DCs (Figure 2D–E). Although both DCs and MFs are among the major producers of BAFF (46, 67–69), we found that transfer of BAFF-producing DCs, but not MFs restored the numbers of GC B cells in $BAFF^{-/-}$ chimeras and $CD16^{-/-}$ mice. This may reflect the tripartite co-localization and interactions between DCs, T cells and B cells during adaptive immune responses. Though they are most well-known for presenting antigen to and activating T cells, DCs also directly modulate B cells through production of IC-induced cytokines like IFN, BAFF and IL-12 (55, 56, 71). Our data do not rule out a role for other immune cell types expressing CD16, like neutrophils, but highlight a key role for DCs as BAFF producers in T-dependent adaptive immune responses.

Because GC B cells, Tfh cells, or memory B cells were not completely absent in the $BAFF^{-/-}$ chimeras or the $CD16^{-/-}$ mice, it is also possible that BAFF from non-hematopoietic cell sources contributes to the response, or that BAFF is not the only factor induced by IgG-ICs that is involved in early GC events. Early in the adaptive immune response, B cells interact with cognate T cells and co-stimulatory signals induce AID activation and class switch (70). These signals include CD40L, which is also required for GC formation (71). We found that $BAFF^{-/-}$ chimeras had normal AID expression and

primary IgG production, suggesting that cognate interactions mediated by CD40L were intact, possibly accounting for the small number of GC and memory B cells that remain despite the absence CD16 or BAFF. It is unlikely that BAFF and CD40L are completely redundant since diminished memory B cell responses were noted in both BAFF$^{-/-}$ chimeras (Figures 1 and 2) and CD40L deficient mice (72, 73).

It is unclear whether the observed role of BAFF occurs before, during, or after the initial interaction between cognate B and T cells. Because AID induction is intact in BAFF$^{-/-}$ chimeras, BAFF likely acts downstream of initial T:B interactions. Our *in vitro* data show that recombinant BAFF directly induced Bcl-6 in activated B cells, and DC CM (elicited under conditions that induce BAFF) diminished the expression of XBP-1 and IRF-4. This is supported by our *in vivo* data where increased numbers of PCs were evident in BAFF$^{-/-}$ chimeras. This suggests that BAFF acts on activated B cells at the time the PC phenotype is being diminished. Therefore, BAFF may act on B cells after cognate T cells help to reduce the PC phenotype, and either commit cells to the memory or GC pathways, or support the survival of precursors that will enter those pathways. This is consistent with studies showing that DC secretion of the PC-inducing cytokine, IL-12, is dampened by IC signaling (74).

Another signal required for a productive GC reaction is IL-21. It is possible that BAFF acts in concert with IL-21 to induce and/or sustain expression of Bcl-6 in GC B cells (75, 76). T cell-secreted IL-21 acts on B cells both during initial T:B interactions and after GCs are formed to promote either Blimp-1 or Bcl-6 expression, depending on the context (77). BAFF may serve as a contextual signal early in the adaptive response to direct B cells toward a GC fate. BAFF may also maintain Bcl-6 in B cells destined for the GC, allowing IL-21 from Tfh cells to take over as the response progresses.

Previous studies showed that cells with a Tfh phenotype appear by day 3 after immunization (10, 78). These cells migrate towards follicles where they interact with B cells at the T-B border (79) to promote continuous expression of Bcl-6, and entry of Tfh cells into the GC (15, 80, 81). Our data suggest that B cells and IgG-ICs are not involved in the formation of Tfh cells (day 3). This is consistent with previous studies showing that on days 1–3 post-immunization, the expression of Bcl-6 and CXCR5 in CD4$^+$ T cells is independent of B cells, and hence independent of IgG (10, 56). Instead, BAFF acted downstream of the formation of Tfh cells, and stabilized and/or expanded the population between days 3 and 7. Thus, previous studies showing a role for B cells in maintaining Tfh cells (22, 78, 82) might reflect the need for B cell-elicited Ig, ICs, and DC-derived BAFF. The role of BAFF in maintaining the Tfh population may be direct, or may involve GC B cells. One possibility is that BAFF promotes the expression of ICOSL on B cells. Previous studies showed that signaling through BAFF-R regulates the expression of ICOSL on B cells (32, 33), thereby sustaining interaction between Tfh cells and B cells at the T:B border and within GCs (10, 11). This interaction could also stabilize the expression of Bcl-6 and the downstream molecules CXCR5 and PD1 (14, 15, 83) to maintain the "Tfh phenotype". This possibility is supported by studies showing that the absence of ICOSL on B cells reduces the frequency of CXCR5$^+$ CD4$^+$ cells after immunization (15), that ICOS:ICOSL interaction prolong the engagement between B cells and Tfh cells (80), and that follicular bystander B cells support

the formation and/or maintenance of Tfh cells by providing ICOSL in an antigen-independent manner (84).

Because the interaction between GC B cells and Tfh cells is bidirectional, the maintenance of Tfh cells may have also had a role in sustaining GC B cells. Recent work described Tfh as a source of local BAFF within GC B cells that is required for affinity maturation (5). Tfh-derived BAFF, however, is not required for GC initiation or maintenance (5), and its role in memory B cell development remains unknown. Our work indicates that early production of BAFF is upstream of GC formation and memory B cell development. Interestingly, we observed a more complete loss of antigen-specific GC B cells (Ac38^{+}) than total GC B cells in BAFF^{−/−} chimeras and CD16^{−/−} mice. This may reflect a loss of local BAFF within the GC due to diminished maintenance and/or expantion of Tfh cells. However, more work would be needed to determine whether affinity maturation is altered in these models.

Overall, our studies highlight a novel role for IgG-ICs and DC-derived BAFF in the GC response. Elucidating the events that initiate GC responses may impact our understanding of ICs and BAFF in autoimmunity. Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by elevated levels of BAFF, autoantibody/autoantigen ICs, and multi-organ pathology. The formation of autoreactive memory is thought to be instrumental in driving long-lived PCs and sustaining autoantibody production (85, 86); however, the mechanisms that regulate memory formation to self-antigens are unclear. Our findings suggest that chronically high levels of ICs containing self-antigens could contribute to a break in B cell tolerance at the GC checkpoint. In SLE, elevated levels of circulating ICs may elevate BAFF and promote the GC response (85, 87, 88). This suggests that neutralization of BAFF in patients with SLE may affect both B cell survival and GC responses that are necessary in the formation of autoreactive B cells, T cell, and memory cells (67, 89, 90).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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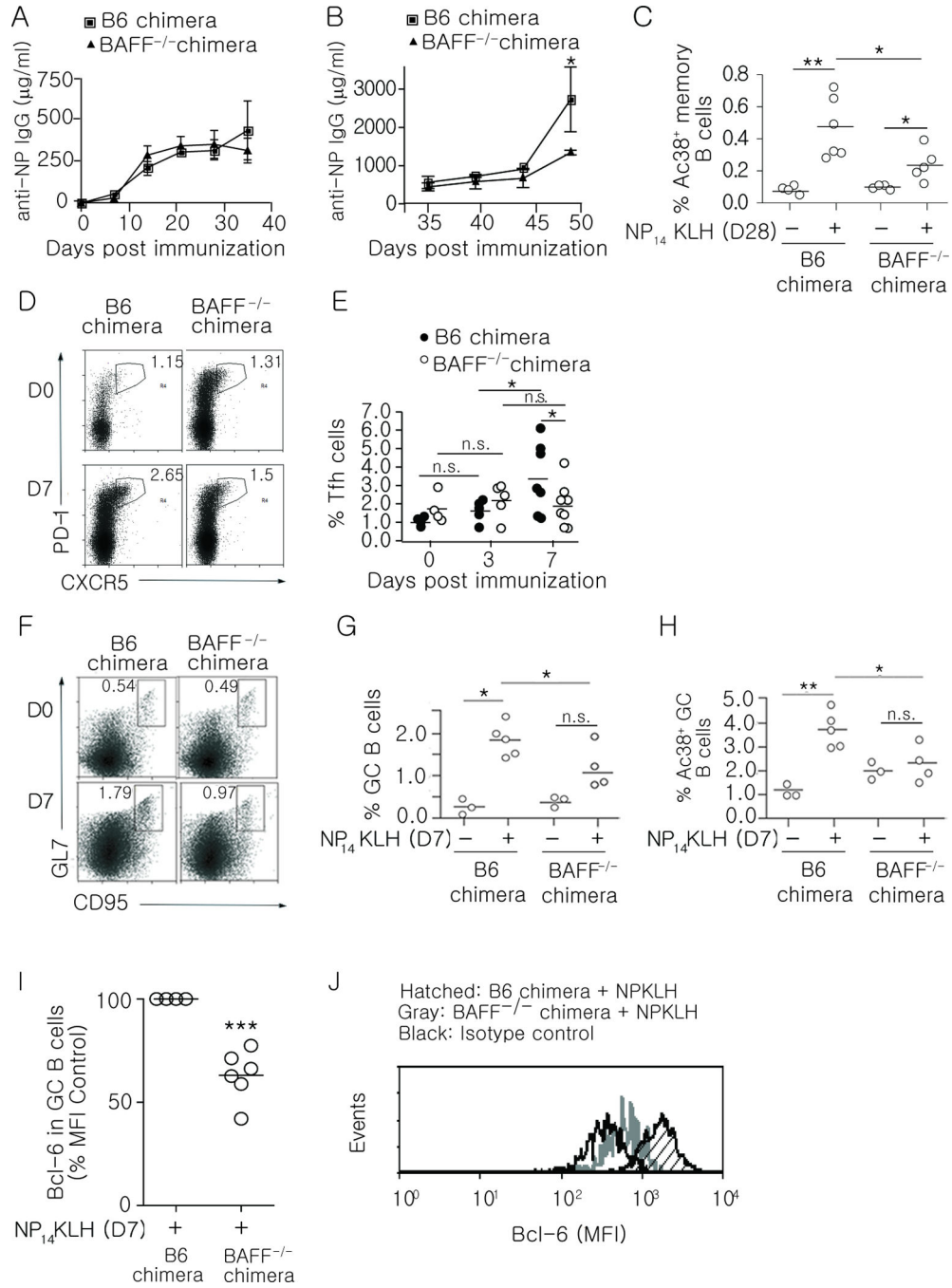
Figure 1. BAFF$^{-/-}$ chimeras have defective memory and GC B cells, diminished Bcl-6 levels and decreased frequency of Tfh cells

(**A**) B6 and BAFF$^{-/-}$ chimeric mice were immunized (i.p) with 100 mg NP$_{14}$KLH in alum. Serum IgG anti-NP responses in B6 and BAFF$^{-/-}$ chimeric mice were measured by ELISA on days 7, 14, 21, 28, and 35 post immunization. n=4–6 mice per group over 3 experiments. (**B**) Serum IgG anti-NP levels measured on days 39, 42, and 49 (4, 7, and 14 days) following boost of 100 mg of soluble NP$_{14}$KLH on day 35. n = 3–6 mice over 3 experiments. (**C**) The frequency of CD19$^+$Ac38$^+$ IgG$^+$ B cells was determined by flow cytometry from the spleens

of B6 and BAFF$^{-/-}$ chimeras immunized for 28 days. The number of splenocytes from B6 and BAFF$^{-/-}$ chimeras was comparable. n = 4–6 mice over 4 experiments. **(D and E)** The frequency of CD4$^+$CXCR5$^+$PD-1$^+$ T cells from B6 and BAFF$^{-/-}$ chimeric mice was measured on days 0, 3 and 7 following immunization. n = 3–6 mice per time point over 2 experiments. **(F and G)** The frequency of CD19$^+$, GL-7$^+$, CD95$^+$ GC B cells was measured by flow cytometry on day 7 post-immunization. n = 3–5 mice over 3 experiments. **(H)** The frequency of CD19$^+$Ac38$^+$CD95$^+$GL-7$^+$ B cells was measured on day 7 post-immunization. n = 3–5 mice over 3 experiments. **(I and J)** Relative expression of Bcl-6 in GC B cells (CD19$^+$CD95$^+$GL-7$^+$) from B6 and BAFF$^{-/-}$ chimeras measured 7 days after NP$_{14}$KLH immunization (i.p.). n = 4–6 mice per group over 2 experiments. Bars display mean **(C, E, G, H, and I)**, error bars indicate standard deviation **(A and B)**. ** p 0.01, *p 0.05, n.s. = not significant.
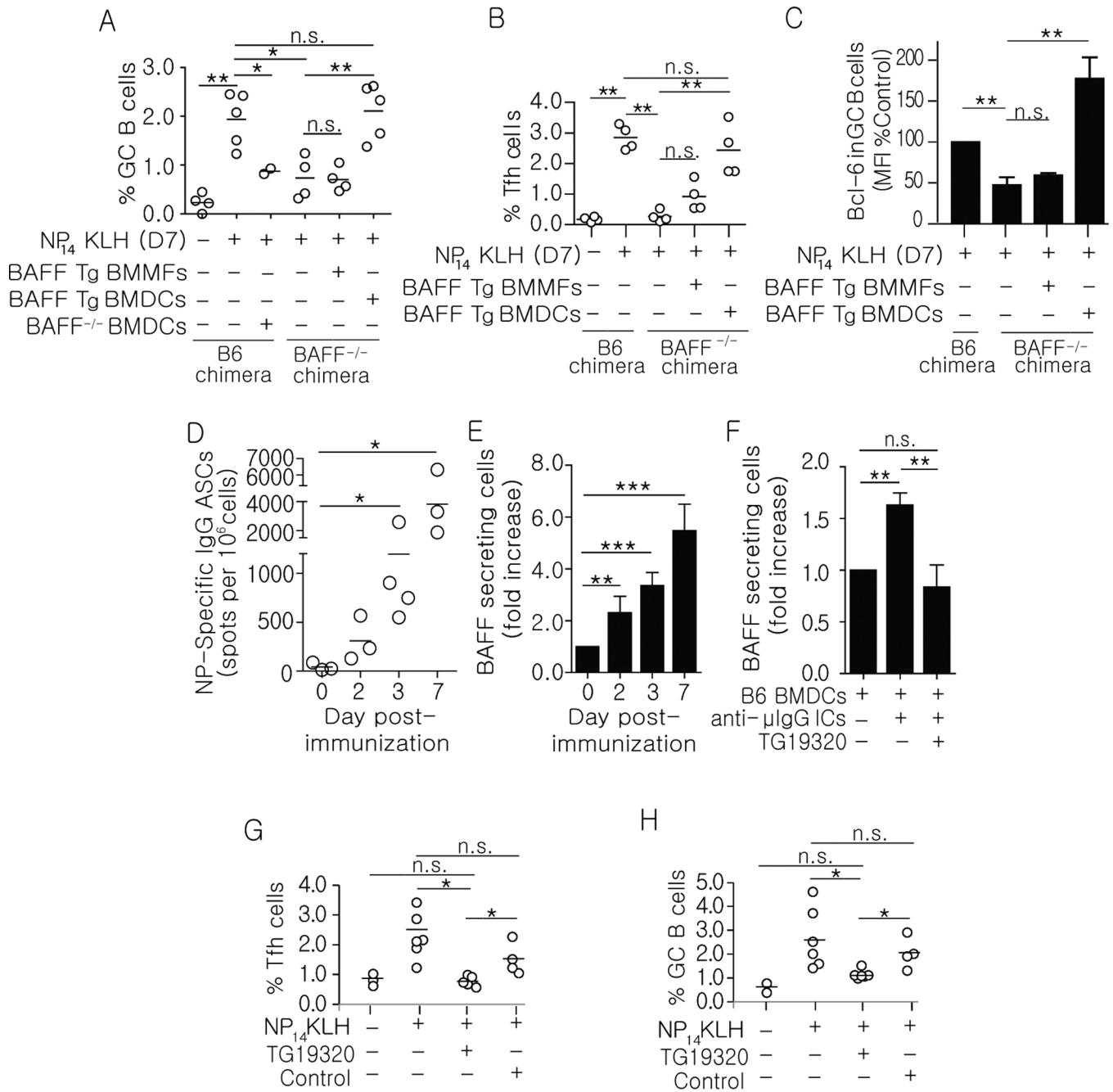
Figure 2. Secretion of BAFF by DCs restores Bcl-6 levels and the frequency of GC B and Tfh cells

(A–C) BAFF Tg BMDCs, BMMFs, or BAFF$^{-/-}$ BMDCs (8×10^6) were injected (s.c.) into B6 or BAFF$^{-/-}$ chimeric mice that were simultaneously immunized (s.c.) with NP$_{14}$KLH. On day 7, inguinal lymph nodes were harvested and the frequencies of **(A)** GC B cells (GL-7$^+$CD95$^+$ cells in CD19$^+$ B cells), **(B)** Tfh cell (CXCR5$^+$PD-1$^+$ cells in CD4$^+$ T cells), and **(C)** Bcl-6 expression in GC B cells from inguinal lymph nodes was measured by flow cytometry. n = 3–5 over 3 experiments. **(D)** On days 2, 3, and 7 following immunization, 1×10^6 splenocytes from B6 mice were plated on NP-BSA-coated ELISpot plates. After 18

hours, NP-specific ASCs were quantitated. n = 3–4 mice per time point over 3 experiments. **(E)** 1×10^6 purified CD11c$^+$ cells from B6 mice immunized for 2, 3, and 7 days were plated on BR3-Fc coated ELISpot plates. After 60 hours, the number of BAFF secreting cells was enumerated. n = 3–6 mice per time point over 3 experiments. BAFF ELISpots ranged from 32-137 (D0-D7). **(F)** BAFF secreting cells enumerated from B6 BMDCs (2.5×10^5) cultured for 24 hrs in the presence or absence of anti-µ ICs, with or without Fc blocking peptide (TG19320; 50 µg/ml). n = 3–10 over 6 experiments. BAFF ELISpots ranged from 531–1051 (untreated and treated with IgG-ICs ± TG19320). **(G–H)** B6 mice were immunized with NP$_{14}$KLH and dosed with 15–30 mg/kg of Fc blocking peptide (TG19320) or an unrelated scrambled peptide (control) via i.p. injection. On day 7, the frequency of Tfh cells (CD4+CXCR5+PD1+, **G**) and GC B cells (CD19+GL7+CD95+, **H**) was determined. n = 3–6 over 2 experiments. Bars display mean **(A, B, D, G, and H)**, and error bars indicate standard error of the mean **(C, E, and F)**. **p 0.01, *p 0.05, n.s. = not significant.
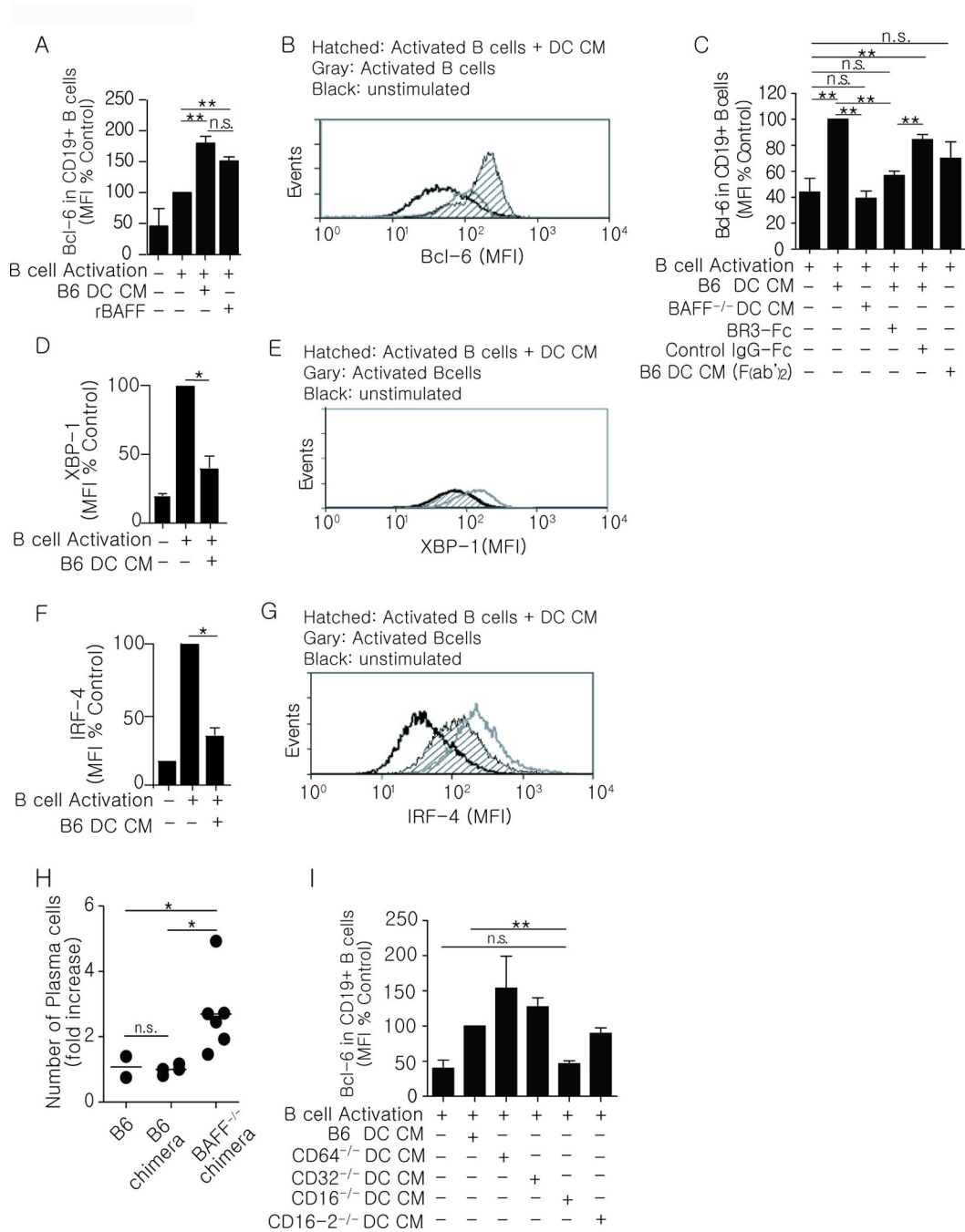
Figure 3. DC-derived BAFF regulates the expression of Bcl-6 in cultured B cells

Purified B6 B cells (1×10^5) stimulated with anti-μ (30 μg/ml), IL-4 (25 ng/ml), and IL-5 (25 ng/ml) (designated as B cell activation) were co-cultured with DC CMs. Intracellular levels of key transcription factors (Bcl-6, XBP-1, IRF-4) were measured by flow cytometry on day 2. **(A)** Bcl-6 expression in B cells co-cultured with B6 DC CM or rBAFF (5 ng/ml). Flow analysis was restricted to live cells. **(B)** Representative histogram of Bcl-6 expression in B cells activated by DC CM as in (A). n = 3–4 mice over 4 experiments. **(C)** Purified B6 B cells were co-cultured with CM generated from B6 BMDCs treated with ICs containing

intact IgG (B6 DC CM) or F(ab')$_2$ of IgG (B6 DC CM (Fab')$_2$), BAFF$^{-/-}$ DC CM, or B6 DC CM neutralized with BR3-Fc (10 μg/ml) or control IgG-Fc (10 μg/ml). n = 4–7 over 4 experiments. **(D–G)** B cells were co-cultured with B6 DC CM. Intracellular levels of XBP-1 **(D, E)** and IRF-4 **(F, G)** were quantitated. n = 3 mice per group over 3 experiments. Histograms are representative of 3 experiments and all flow analysis was done after gating on live cells. **(H)** Numbers of splenic plasma cells (CD138+B220−) were enumerated by flow cytometry in B6, B6 chimera, or BAFF$^{-/-}$ chimeras at day 5 following immunization. n = 1–3 mice per group over 2 experiments. **(I)** Bcl-6 expression in B cells cultured with DC CM from B6, CD64$^{-/-}$, CD32$^{-/-}$, CD16$^{-/-}$, or CD16-2$^{-/-}$ (FcγRIV) mice. n=6–7 mice per group over 4 experiments. Bars display mean **(H)**, and error bars indicate standard error of the mean **(A, C, D, F, and I)**. **p   0.01, * p   0.05, n.s. = not significant.
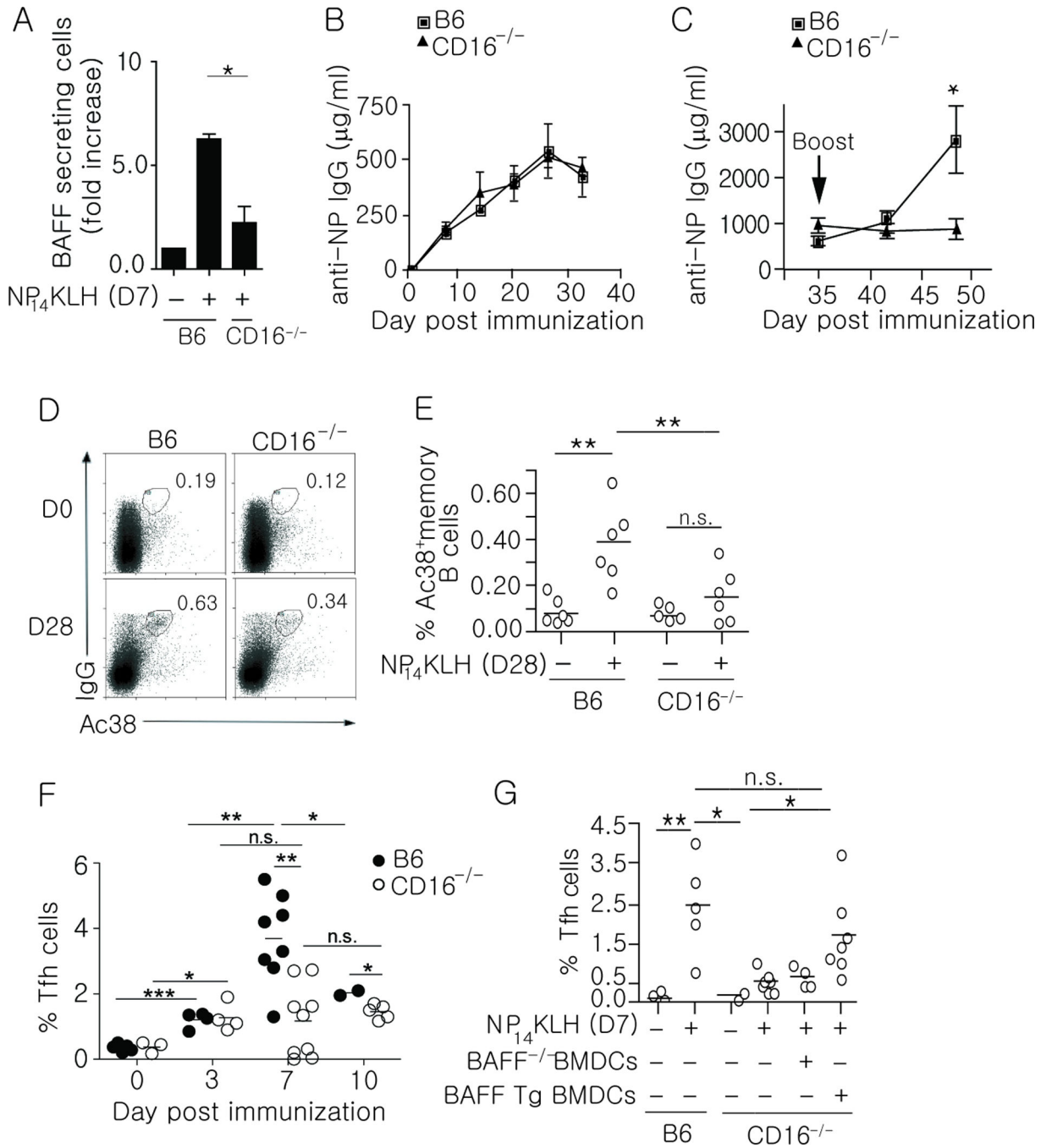
Figure 4. CD16$^{-/-}$ mice have defective BAFF secretion, secondary antibody responses, frequency of memory B cell and Tfh cells

(**A**) BAFF secreting cells enumerated from purified CD11c$^+$ cells that were isolated from B6 and CD16$^{-/-}$ mice on day 7. n = 3 over 3 experiments. B6 and CD16$^{-/-}$ mice were immunized (i.p) with 100 mg NP$_{14}$KLH in alum. Serum IgG anti-NP (**B**) primary responses measured by ELISA on days 0, 7, 14, 21, 28, and 35; serum IgG (**C**) secondary antibody levels measured on days 39, 42, and 49 (days 4, 7, and 14) following boost of 100 mg soluble NP$_{14}$KLH on day 35. n = 4–8 mice over 3 experiments. (**D and E**) The frequency of

CD19$^+$Ac38$^+$IgG$^+$ memory B cells was determined by flow cytometry from the spleens of B6 and CD16$^{-/-}$ mice immunized for 28 days. The number of splenocytes in B6 and CD16$^{-/-}$ mice was comparable. n = 5–6 mice over 3 experiments. Data are expressed as the percent of CD19$^+$ cells that are IgG$^+$ and Ac38$^+$. **(F)** The frequencies of CD4$^+$CXCR5$^+$PD-1$^+$ T cells from immunized B6 and CD16$^{-/-}$ mice on days 0, 3, 7, or 10. n = 2–8 mice per time point over 4 experiments. **(G)** B6, BAFF Tg, and BAFF$^{-/-}$ BMDCs (8 $\times$ 10^6) were injected into CD16$^{-/-}$ mice that were simultaneously immunized with NP$_{14}$KLH. On day 7, the frequencies of CD4$^+$CXCR5$^+$PD-1$^+$ T cells from inguinal lymph nodes were enumerated by flow cytometry. n = 3–7 mice over 3 experiments. Bars display mean **(E, F, and G)**, and error bars indicate standard error of the mean **(A)** or standard deviation **(B and C)**. **p 0.01, *p 0.05, n.s. = not significant.
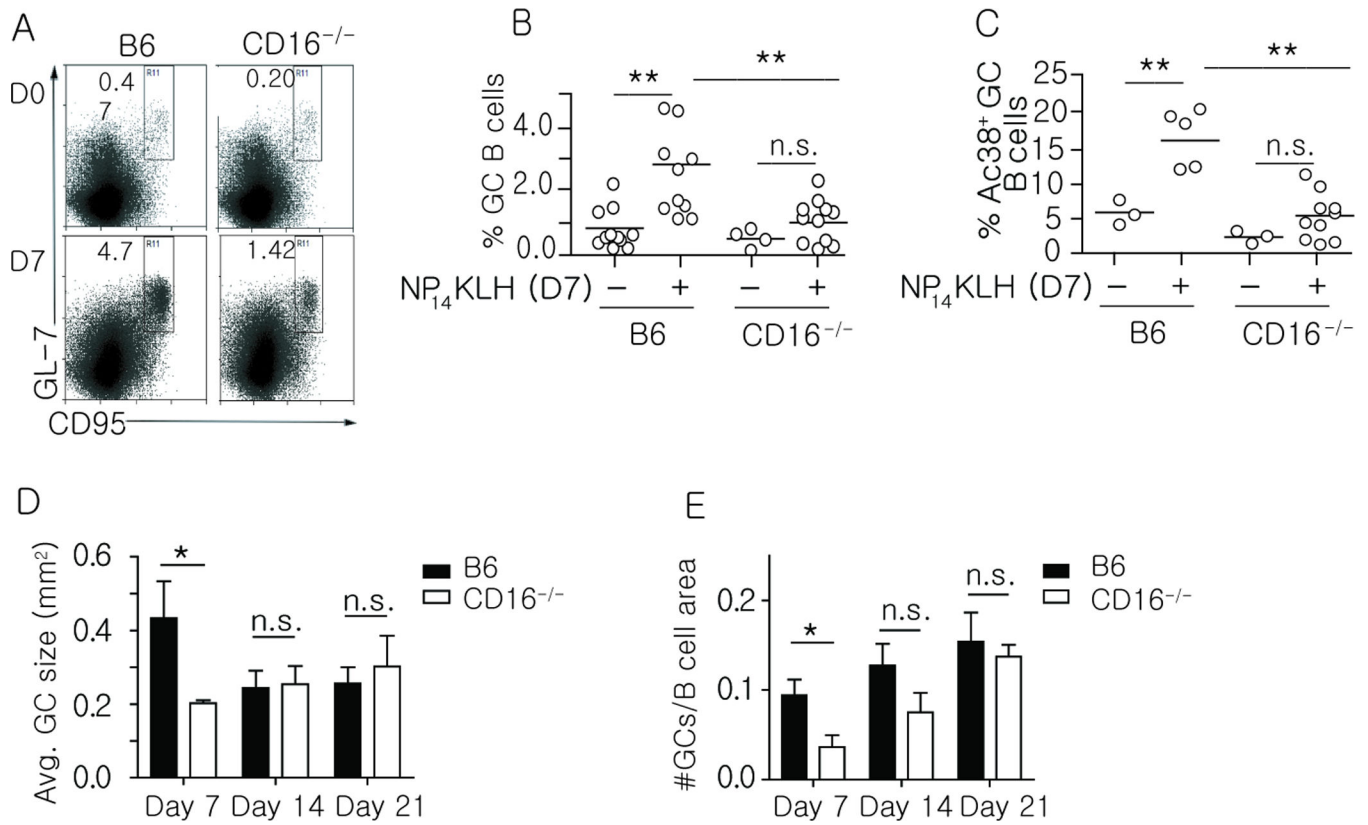
Figure 5. CD16$^{-/-}$ mice have diminished germinal center responses
(A and B) The frequency of splenic CD19$^+$GL-7$^+$CD95$^+$ GC B cells was measured by flow cytometry from B6 and CD16$^{-/-}$ mice 7 days after immunization. n = 4–11 mice per group over 3 experiments. **(C)** The frequency of NP-specific GC B cells (CD19$^+$Ac38$^+$GL-7$^+$CD95$^+$) measured in B6 and CD16$^{-/-}$ mice 7 days after immunization. n = 3–10 mice over 3 experiments. **(D)** The size of GCs and **(E)** the number of GCs were enumerated from B6 and CD16$^{-/-}$ mice 7, 14, and 21 days after immunization using confocal microscopy. n = 3–6 mice per time point over 4 experiments. Bars display mean **(B and C)**, and error bars indicate standard error of the mean **(D and E)**. **p   0.01, *p   0.05, n.s. = not significant.
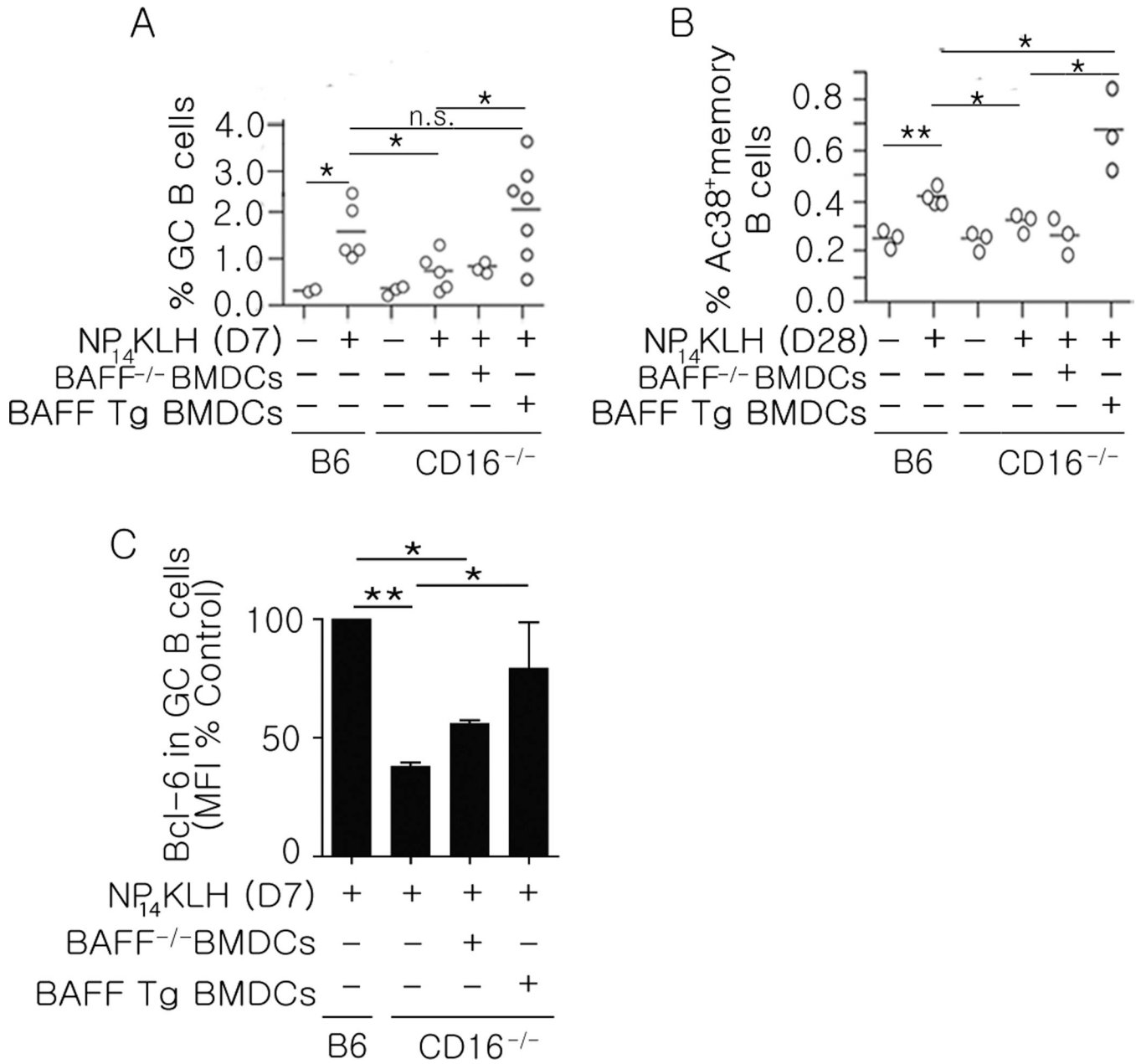
Figure 6. Adoptive transfer of BAFF expressing DCs rescues GC and memory B cell populations and restores Bcl-6 levels in CD16$^{-/-}$ mice B cells

(A) BMDCs (8×10^6) from BAFF Tg or BAFF$^{-/-}$ mice were injected (s.c.) into CD16$^{-/-}$ mice and simultaneously immunized (s.c.) with NP$_{14}$KLH. On day 7, the frequency of GC B cells (CD19$^+$GL-7$^+$CD95$^+$) were measured from inguinal lymph nodes. n = 3–7 mice per group over 3 experiments. (B) Same as (A) but on day 28, the frequency of CD19$^+$Ac38$^+$IgG$^+$ memory B cells was measured. n = 3–4 over 2 experiments. (C) BAFF Tg and BAFF$^{-/-}$ BMDCs (8×10^6) were injected into CD16$^{-/-}$ mice and simultaneously immunized with NP$_{14}$KLH. n = 4 mice over 3 experiments. On day 7, Bcl-6 expression in GC B cells from inguinal lymph nodes was measured by flow cytometry. Bars display

means **(A and B)**, and error bars indicate standard error of the mean **(C)**. **p 0.01, *p 0.05, n.s. = not significant.