Constitutive CD40L Expression on B Cells Prematurely Terminates Germinal Center Response and Leads to Augmented Plasma Cell Production in T Cell Areas

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Constitutive CD40L Expression on B Cells Prematurely Terminates Germinal Center Response and Leads to Augmented Plasma Cell Production in T Cell Areas

Anna Bolduc,* Eugene Long,* Dale Stapler,* Marilia Cascalho,† Takeshi Tsubata,‡ Pandelakis A. Koni,* and Michiko Shimoda*

CD40/CD40L engagement is essential to T cell-dependent B cell proliferation and differentiation. However, the precise role of CD40 signaling through cognate T–B interaction in the generation of germinal center and memory B cells is still incompletely understood. To address this issue, a B cell-specific CD40L transgene (CD40LBTg) was introduced into mice with B cell-restricted MHC class II deficiency. Using this mouse model, we show that constitutive CD40L expression on B cells alone could not induce germinal center differentiation of MHC class II-deficient B cells after immunization with T cell-dependent Ag. Thus, some other MHC class II-dependent T cell-derived signals are essential for the generation of germinal center B cells in response to T cell-dependent Ag. In fact, CD40LBTg mice generated a complex Ag-specific IgG1 response, which was greatly enhanced in early, but reduced in late, primary response compared with control mice. We also found that the frequency of Ag-specific germinal center B cells in CD40LBTg mice was abruptly reduced 1 wk after immunization. As a result, the numbers of Ag-specific IgG1 long-lived plasma cells and memory B cells were reduced. By histology, large numbers of Ag-specific plasma cells were found in T cell areas adjacent to Ag-specific germinal centers of CD40LBTg mice, temporarily during the second week of primary response. These results indicate that CD40L expression on B cells prematurely terminated their ongoing germinal center response and produced plasma cells. Our results support the notion that CD40 signaling is an active termination signal for germinal center reaction. The Journal of Immunology, 2010, 185: 220–230.

CD40 is a member of the TNFR family and is constitutively expressed on B cells. CD40L is a member of the TNF ligand family and is expressed on activated CD4+ T cells. CD40/CD40L engagement triggers activation of the canonical and the noncanonical NF-κB–signaling pathways and promotes proliferation and survival of B cells (1, 2). CD40/CD40L engagement during cognate T–B interaction through MHC class II (MHC-II)-restricted Ag presentation is critical to T cell-dependent B cell differentiation, including Ig class-switching, germinal center differentiation, and subsequent memory B cell and long-lived plasma cell generation (3, 4).

In addition to activated T cells, CD40L is expressed on other cells, such as monocytes (5), platelets (6), and lung fibroblasts (7), upon inflammation. CD40L delivered in the form of platelet-derived membrane vesicles was shown to stimulate Ag-specific IgG production and modulate germinal center formation through cooperation with responses elicited by CD4+ T cells (8). Furthermore, aberrant CD40L expression on B cells has been observed in systemic lupus erythematous patients (9), lupus-prone BXSB mice (10), and B cell lymphoma (11). Thus, CD40-derived signaling in B cells due to interactions with non-T cells or aberrant CD40L expression on B cells can alter B cell function and differentiation in certain inflammatory conditions and, thereby, might regulate the development and progression of certain diseases. In fact, B cell-restricted CD40L transgenic (CD40LBTg) mice develop lupus-like disease (12) or colitis (13) with age in association with autoantibody production. However, the mechanisms by which the autonomous CD40 signal on B cells triggers autoimmunity are not entirely clear. It was shown that autonomous CD40 signaling enhances B cell survival and protects activated B cells from apoptosis (12, 14). It is also speculated that T cell-independent CD40/CD40L signals may sufficiently replace some T cell functions, such as induction of germinal center differentiation and memory B cell generation, which might accelerate autoimmunity.

We previously generated B cell-specific MHC-II–deficient IA-B mice (15), which lack MHC-II expression on B cells due to deletion of a conditional Iab allele. Using IA-B mice, we previously showed that MHC-II expression on B cells is essential for germinal center B cell differentiation in response to T cell-dependent Ag (15) (i.e., the early primary response in IA-B mice was severely impaired because of B cell-restricted MHC-II deficiency but recovered to a level similar to that of wild type (WT) mice in association with dramatic clonal expansion and germinal center differentiation exclusively from the <5% of Ag-specific B cells that still expressed MHC-II due to incomplete cd19-cde-driven deletion) (15). In this study, we introduced a B cell-specific CD40L transgene onto the IA-B mouse background by intercrossing with a transgenic (Tg) mouse line expressing CD40L on B cells under the control of the...
IgNH1 promoter, IgH enhancer, and 3’Igk enhancer (CD40LTg mice) (12). Using this new IA-B/CD40LTg mouse model, we addressed whether autonomic CD40/CD40L signaling on B cells could sufficiently replace cognate T cell help and induce germinal center differentiation of MHC-II–deficient B cells.

We found that T cell-dependent Ag-specific IgG1 production in IA-B/CD40LTg and CD40LTg mice was greatly increased in the early primary response, but it was reduced in the late primary response. We show that autonomic CD40 signaling alone could not induce germinal center B cell differentiation of MHC-II–deficient B cells, by in vivo cellular analysis of germinal center B cell differentiation in IA-B/CD40LTg mice and adoptive transfer experiments. Therefore, the enhanced early primary response was not caused by MHC-II–independent germinal center generation due to CD40L expression. In fact, in immunized CD40LTg mice, germinal center development was reoriented at day 7 postimmunization, which resulted in temporal production of large numbers of Ag-specific plasma cells in T cell areas. Our results indicated that enforced CD40 signaling has the potential to increase plasma cell production by prematurely terminating Ag-specific MHC-II–dependent germinal center reaction.

**Materials and Methods**

**Mice**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). All of the mice used in this study were on a C57BL/6 background (n > 10) and were bred in our facility under specific pathogen-free conditions. Mice with a conditional loxP-targeted IA-B chain (iabneo) allele were described previously (16). Mice that lack MHC-II specifically from B cells (IA-B mice) were generated by interbreeding iabneo mice with cd19cre mice (15). Mice were genotyped for cre by PCR with primers described elsewhere (17). Cell-specific MHC-II deletion was confirmed by analyzing B cells in peripheral blood by flow cytometry at 6 wk of age.

CD40LTg mice were generated elsewhere (12). Unlike another CD40LTg mouse line that spontaneously develops colitis with age (13), the CD40LTg mouse line that we used in this study is less susceptible to develop colitis or lupus-like disease under the specific pathogen-free conditions of our facility. CD40LTg mice were crossed onto IA-B background (15) to generate IA-B/CD40LTg mice. The presence of the transgene was identified by tail DNA PCR assays. Typical experiments used 8–12-wk-old C57BL/6 mice and CD40LTg mice. For studying humoral responses, 7–10-wk-old iabneo cd19cre mice (IA-B mice), IA-B/CD40LTg mice, iabneo+cd19cre mice, and iabneo+cd19cre+CD40LTg mice were used for immunization. For some experiments, quasi-monoclonal (QM) Tg mice (18) crossed on each genetic background were used.

**Immunization**

(4-Hydroxy-3-nitrophenyl)acetyl (NP)13–chicken γ-globulin (CGG) was prepared as described elsewhere (15). Mice 7–10 wk of age were given an i.p. challenge with 100 µl PBS containing 50 µg NP13–CGG absorbed in alum or 50 µg NP23–Ficoll (Biosearch Technologies, Novato, CA). Blood was collected from immunized mice by tail vein bleeding for serum Ab determination at various time points. All studies were reviewed and approved by an appropriate institutional committee.

**Abs and reagents**

Abs used in this study were from BD Biosciences (San Jose, CA), unless otherwise indicated, and included Abs against IA (AF6-120.1), CD4 (RM4-5), CD11c (HL3), CD11b (M1/70), CD5 (53-7.3), B220 (RA3-6B2), IgM (R6-20.2), CD21/CD35 (7G6), CD23 (B384), CD24 (M1/69), IgD (217-170), CD8 (53-6.7), CD90.1 (HIS51), TCR (H57-597), CD45R (CD11c), CD38 (281-2), 2.4G2; anti-CD16/CD32, CD43 (57), Gr-1 (RB6-8C5), CD49d (DX5), CD90.2 (30-H12), ICOS ligand (ICOS-L) (HK5.3), CD80 (16-10A1), CD86 (GL1), CD40L (MR1; eBioscience), CD95 (15A7; eBioscience), CD21R (44A9; eBioscience), OX40L (252; BioLegend), and CD38 (90; eBioscience). PNA-FITC was from Vector Laboratories (Burlingame, CA). NP-haptenated PE (NP23–PE) was prepared as described elsewhere (15).

**Flow cytometry and cell sorting**

Single-cell suspensions were prepared from spleens following the depletion of RBCs with ACK lysing buffer (BioWhittaker, Walkersville, MD). Spleen cells were then washed twice with PBS and filtered through nylon mesh in RPMI 1640/1% FCS. For general analysis, cells were pretreated with eBioscience anti-CD16/CD32 Ab on ice for 15 min and then incubated on ice for 15–30 min with specific Abs. Cells were analyzed on a FACSCalibur (BD Biosciences) with CellQuest software (BD Biosciences), Canto (BD Biosciences) with Diva software, or MoFlo with Summit software version 4.0 (both from DakoCytomation).

Ag-specific germinal center B cells and B220+ memory B cells were identified by five-color analysis, as previously reported. Briefly, cell-bound Ig was stripped by incubating spleen cells at 37°C for 30 min, followed by washing twice with PBS/1% FCS before staining with specific Abs on ice for 20 min (19). After incubation with Fc block (2.4G2), a mixture of bio-tinylated Abs to IgM, IgD, CD43, CD5, Gr-1, CD11b, CD49d, and CD90.2 was used to exclude naive B cells, plasma cells, B-1 cells, macrophages, NK cells, and T cells (lineage-depletion Ab mixture). For some experiments, NP-specific IgG1 memory B cells were identified as IgG1+ NP-binding cells among B220+ cells. After washing, cells were stained with anti–Ig1-FITC, NP23–PE, streptavidin–PE-Cy5, anti-CD38–allophycocyanin, and B220–allophycocyanin-Cy7. Cells were washed and finally suspended in 5 µg/ml 7-aminocyclohexane D (Sigma-Aldrich, St. Louis, MO) for analysis. At least 2 million events were collected for memory B cell analysis.

Marginal zone (MZ) B cells (CD21+CD23−B220+) and follicular (FO) B cells (CD21−CD23+ B220+) were sorted from spleen cells using an Aria cell sorter.

**Cell culture**

Sorted MZ B cells and FO B cells were stained with 5 µM CFSE (Invitrogen, Carlsbad, CA), plated into a 96-well plate (200 µl/well) or a 48-well plate (400 µl/well) in RPMI 1640/10% FCS at a concentration of 0.5–1 × 106/ml and cultured for various time points at 37°C with 5% CO2 in the presence of various stimuli, such as 5–10 µg/ml anti-CD40 mAb (HM40-3), goat anti-mouse IgM Fab fragments (Jackson ImmunoResearch Laboratories, West Grove, PA), CyP oligodeoxynucleotide (ODN) 1826, LPS (Escherichia coli 0111:B4, Sigma-Aldrich), or 100 U/ml IL-4 (PeproTech, Rocky Hill, NJ).

**Adoptive transfer**

MHC-II− or MHC-II+ MO B cells and FO B cells were purified from pooled spleens by Aria or magnetic cell sorting with a B cell purification kit (Miltenyi Biotec, Auburn, CA). FO B cells (2 × 106) or MZ B cells (2 × 106) were mixed with carrier B cells from iabneo cd19cre mice (2 × 106) and injected i.v. into iabneo cd19cre mice or C57BL/6 CD45.1 mice. Twenty-four hours later, mice were challenged i.p. with 50 µg NP-CGG with alum. We estimated the relative frequency and consistency of donor cell transfer based on CD45.2 expression among B220+ cells in peripheral blood cells 1 d after transfer. Typically, ~1.5% of B220+ cells in peripheral blood were CD45.2+ cells after transfer (data not shown). Anti-NP response was analyzed by ELISA and FACS 7–14 d after the immunization.

**ELISA and ELISPOT assay**

ELISA and ELISPOT assay were performed as previously described (15). Total and high-affinity NP-specific Abs in sera were measured using a Clono-typing System/HRP (Southern Biotechnology Associates, Birmingham, AL) with tetramethylbenzidine substrate, using plates coated with NP13–BSA and NP23–BSA, respectively. The reciprocal end point titer for total (NP13–BSA) and high-affinity (NP23–BSA) NP-specific Abs in sera was defined as the dilution of serum giving an OD of 0.05 at 405 nm. Pooled sera from NP-CGG–immunized control mice between 12 and 20 wk postimmunization were used as a standard control. Similarly, total and high-affinity NP-specific Ab-secreting cells in spleen and bone marrow were determined by 3-h culture on plates coated with NP13–BSA and NP23–BSA, respectively, followed by detection with a Clonotyping System/AP as above, but with a Liquid BCP/NBT Substrate Kit (Zymed Laboratories, San Francisco, CA).

**Histology**

Spleens were snap-frozen in Optimal Cutting Temperature compound in a dry ice/methylbutane bath. Frozen spleen tissue sections of 7-µm thickness were prepared with a cryostat microtome (Leica Microsystems, Deerfield, IL), fixed in cold acetone for 20 min, air-dried, and stored at −80°C until staining. Thawed serial sections were rehydrated in PBS and then preblocked with 1% FCS/PBS before being incubated with anti-I-Ab–PE, NP23–PE, anti-TCRβ, anti-CD138–PE, or anti-CD23–PE and bioin-

**Downloaded from** http://www.jimmunol.org/
anti-IgD, followed by streptavidin Alexa 488 (Molecular Probes, Eugene, OR) or PNA-FITC. Slides were coverslipped before imaging with a Nikon eclipse 90i digital microscopy system (Nikon, Melville, NY).

**Quantitative RT-PCR**

RNA was prepared using a Qiagen Mini Kit (Qiagen, Valencia, CA), according to the manufacturer’s protocol. cDNA was prepared using a cDNA synthesis kit from SABioscience. Quantitative RT-PCR (QRT-PCR) analysis was performed using primers from SABioscience with an iQ5 cycler (Bio-Rad, Hercules, CA). Gene expression levels in each sample were normalized against β-actin expression and statistically analyzed with software from SABioscience/Qiagen (Frederick, MD).

**Western blotting**

A total of 2 × 10⁶ cells were lysed in 20 μl lysis buffer, and cell lysates were prepared on a SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane. P100, p52, IκB-α, and phosphorylated IκB-α were detected by specific Abs against each protein from Cell Signaling Technology (Beverly, MA), followed by HRP-conjugated goat anti-rabbit Ab from Santa Cruz Biotechnology (Santa Cruz, CA). Signals were visualized with ECL Western blotting detection reagents. The amount of loaded protein was standardized against β-actin. After luminescence acquisition with a Fujifilm LAS-3000 (Fujifilm, Valhalla, NY), quantification of bands was performed with Multi Gauge software (Fujifilm). Protein expression values of freshly isolated control FO or MZ B cells were set to 1 to achieve comparability among different Western blots.

**Statistics**

The p values were determined by using the two-tailed Student t test.

**Results**

**Augmented canonical NF-κB activation in CD40L Tg B cells upon anti-CD40 stimulation**

CD40L Tg mice were previously generated to test the impact of autonomous constitutive CD40 signaling on B cells, and these mice proved to develop B cell-intrinsic autoimmunity (12). However, freshly isolated B cells from CD40LTg mice do not have a spontaneously activated phenotype, based on their normal expression levels of CD86 and CD23 (12). Also, CD40L expression on CD40LTg B cells was not detectable unless cells were treated with anti-CD40 Ab, because CD40L constitutively bound to CD40 (12) (Fig. 2C). Thus, it was not clear how constitutive CD40L expression leads to B cell abnormality in this CD40LTg mouse model. Therefore, we first examined NF-κB activation in control and CD40L Tg B cells based on the amount of IκB, phosphorylated IκB (pIκB), p100, and p52 protein before and after anti-CD40 Ab incubation. CD40/CD40L engagement activates canonical and noncanonical NF-κB pathways. The canonical pathway induces phosphorylation of IκB proteins through inhibitor of nuclear factor κ-B kinase subunit α1 (20).

Freshly isolated MZ and FO B cells from CD40L Tg mice expressed a similar amount of IκB and pIκB compared with control B cells. As shown in Fig. 1, upon incubation with anti-CD40 Ab, pIκB in MZ and FO B cells from CD40LTg mice peaked at an earlier time point (~10 h) than it did in control MZ and FO B cells. In the meantime, IκB in control and CD40LTg B cells was decreased with similar kinetics. Expression levels of p100 and p52 were similar in freshly isolated MZ and FO B cells from control and CD40LTg mice. After incubation with anti-CD40 Ab, p52 and p100 in MZ and FO B cells increased with similar kinetics in CD40LTg mice and control mice (Fig. 1). Collectively, our data suggest that activation of the canonical NF-κB pathway in CD40LTg B cells occurred with faster kinetics compared with that in control B cells after anti-CD40 Ab treatment.

CD40L Tg B cells constitutively express a higher level of prdm1 compared with control B cells

To further examine the impact of CD40L expression on B cells, we next performed QRT-PCR analysis for a set of gene transcripts which are known to regulate B cell differentiation into germinal center and plasma cells, before and after stimulation with anti-IgM+IL-4+anti-CD40 and CpG ODN. Consistent with previous reports, freshly isolated control MZ B cells expressed greater levels of prdm1 (~6-fold) compared with control FO B cells (data not shown). Freshly isolated CD40LTg MZ B cells expressed increased levels of prdm1 (3.73-fold; p < 0.001) and irf4 (2.16-fold; p < 0.01) compared with control MZ B cells (Fig. 2). In contrast, prdm1 expression level of CD40LTg FO B cells was also relatively higher than that of control FO B cells, although the difference was not statistically significant. Upon stimulation with anti-IgM+IL-4+anti-CD40 or CpG ODN, MZ and FO B cells from control and CD40LTg mice showed a similar pattern of expression for these genes.

**Generation of mice with B cell-restricted MHC-II deficiency and constitutive CD40L expression**

To examine the impact of autonomous CD40/CD40L signaling, apart from cognate T cell–B cell interaction, we crossed CD40LTg mice (12) onto a B cell-restricted MHC-II–deficient IA-B (Iα-neo/neo cd19 cre/+ background) (15) to generate IA-B/CD40LTg mice (Fig. 3A). Thus, as shown in Fig. 3B, B cells in IA-B/CD40LTg mice lack MHC-II expression due to cd19-cre–mediated conditional deletion of MHC-II (15). As previously described, CD40L expression was not readily detected on freshly isolated B cells from CD40LTg mice by FACS (12). Upon incubation with anti-CD40 Ab, CD40L expression on B cells from CD40LTg mice, but not from control mice, became evident (Fig. 3C).

It was reported that CD40LTg mice show increased B cell number and splenomegaly (12). IA-B/CD40LTg mice also showed significantly enlarged spleen and increased numbers of total spleen cells (by 3–5-fold) at 8 wk of age. Also, like CD40LTg mice, IA-B/CD40LTg mice had an increased frequency of B220⁺ B cells (70–80% of total spleen cells) and a reduced frequency of T1 B cells (CD23⁻CD21⁺B220⁺ cells) compared with non-Tg controls (Fig. 3D). We also confirmed that like CD40LTg B cells, B cells in IA-B/CD40LTg mice were not spontaneously activated based on various cell surface markers (i.e., freshly isolated MZ B cells [CD23⁻ CD21⁺B220⁺] and FO B cells [CD23⁻CD21⁺B220⁺] cells) from CD40LTg mice or IA-B/CD40LTg mice expressed similar levels of CD86, CD23, ICOS-L, OX40L, CD95, and CD21R by FACS compared with their non-Tg counterparts [data not shown]. B cells from CD40LTg mice expressed a slightly increased level of MHC-II compared with B cells from non-Tg littermates (Fig. 3B).

**B cell-specific CD40L transgene expression enhanced early primary, but impaired late primary, humoral response**

We previously showed that the T cell-dependent early primary response in B cell-specific MHC-II–deficient IA-B mice was significantly impaired as a result of B cell-specific MHC-II deficiency, whereas the T cell-independent response was normal (15). We also showed that the impaired early primary response in IA-B mice recovered to a level similar to that of WT mice in association with dramatic clonal expansion and germinal center differentiation from the ~5% Ag-specific B cells that still expressed MHC-II due to incomplete cd19-cre–driven deletion (15). We used IA-B/CD40LTg mice to test whether autonomous CD40/CD40L signaling on B cells could sufficiently replace cognate T cell help. If our hypothesis was correct, IA-B/CD40LTg mice might show an
enhanced or recovered primary response compared with IA-B mice, perhaps in association with germinal center differentiation of MHC-II–deficient B cells.

First, we tested the effect of autonomous CD40/CD40L signaling on the T cell-independent response. After immunization with NP-Ficoll, IA-B/CD40LBTg mice showed \( \sim 10 \) fold increased IgG3 and IgM anti-NP Ab production compared with WT mice (Fig. 4A). Thus, CD40L expression on B cells significantly enhanced plasma cell differentiation upon stimulation with T cell-independent Ag.

After immunization with T cell-dependent Ag NP-CGG, anti-NP IgM titers in IA-B/CD40LBTg mice at 4 and 7 d postimmunization were increased by \( \sim 5 \) fold compared with IA-B mice (Fig. 4B), suggesting that the early extrafollicular IgM response by MZ B cells was enhanced by autonomous CD40L expression. Furthermore, as shown in Fig. 4C, after immunization with T cell-dependent Ag NP-CGG, anti-NP IgG3 titers in IA-B/CD40LBTg mice at 4 and 7 d postimmunization were increased by \( \sim 2.5 \) fold compared with IA-B mice (Fig. 4C), suggesting that the early extrafollicular IgG3 response by MZ B cells was enhanced by autonomous CD40L expression.

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FIGURE 1. Enhanced NF-κB activation in B cells of CD40LBTg mice. MZ and FO B cells from WT control or CD40LBTg mice (CD40L) were stimulated with anti-CD40 Ab (10 μg/ml) for the indicated time periods, and cell lysates were analyzed by Western blotting with specific Abs against pIkBα, IkBα, p100, and p52. A, Representative blot images with β-actin control staining. B, Fold change expression level of pIkBα, IkBα, p100, and p52 in MZ and FO B cells before (0 h) and after stimulation with anti-CD40 Ab at each time point, compared between WT control (○) and CD40LBTg (●) mice. Each protein expression level was normalized based on β-actin staining and compared with the level before stimulation (0 h: 1).

FIGURE 2. QRT-PCR analysis of genes responsible for B cell differentiation. Purified MZ B cells and FO B cells from control WT mice or CD40LBTg mice were cultured in vitro with a combination of IL-4, anti-IgM [F(ab)₂], and anti-CD40 Ab or with CpGODN for 40 h and lysed for preparation of cDNA. QRT-PCR analysis was performed with an IQ5 cycler using primers for indicated genes. Statistical analysis of gene-expression profiles from at least three experiments using β-actin expression as internal control is shown.

FIGURE 3. B cell-specific MHC-II–deficient CD40L Tg mice as a model to study the effect of autonomous CD40/CD40L signaling in the absence of cognate T–B interaction. A, Possible T–B interaction of B cells from WT, CD40LBTg, IA-B, and IA-B/CD40LBTg mice. B, Representative FACS profiles of MHC-II (IA-b) expression on B220+ B cells from WT, CD40LBTg, IA-B, and IA-B/CD40LBTg mice at 8 wk of age. Mean fluorescent intensities of IA-b expression for WT and CD40LBTg mice are shown. C, CD40L expression on MZ and FO B cells from IA-B mice (IA-B) and IA-B/CD40LBTg mice (IA-B/CD40L) was analyzed after stimulation with anti-CD40 Ab (10 μg/ml) for 18 h. Representative graphs of CD40L expression level with (shaded graph) or without (solid line) stimulation compared with isotype control (dotted line) are shown. D, Representative FACS profiles of B220+ B cells from WT, CD40LBTg, IA-B, and IA-B/CD40LBTg mice at 8 wk of age. The frequencies of T1 (CD21⁺CD23⁻), MZ (CD21⁺CD23⁻), and FO (CD21⁺CD23⁺) cells among B220+ cells are shown.
CGG, CD40LBTg mice and IA-B/CD40LBTg mice showed ∼10-fold higher anti-NP IgG1 titers during the early (1–4 wk) primary response compared with WT and IA-B mice, respectively. Thus, overall, the autonomous CD40/CD40L signal reversed the impaired early primary response of IA-B mice. Unexpectedly, however, although the NP IgG1 titers of IA-B mice kept increasing to the level of WT mice in association with delayed germinal center reaction, those of IA-B/CD40LBTg mice were greatly reduced (∼20-fold) compared with IA-B mice in the late (10–20 wk) primary response (Fig. 4C). In addition, as shown in Fig. 4D, anti-NP IgG1 serum Ab affinity maturation (ratio of anti-NP IgG1 titers captured with NP2-BSA and NP15-BSA by ELISA) at 20 wk postimmunization was significantly reduced in CD40LBTg mice and IA-B/CD40LBTg mice compared with their controls.

**Early termination of germinal center B cell response in mice expressing CD40L on B cells**

To understand the mechanisms of the unexpected T cell-dependent humoral response in IA-B/CD40LBTg mice, we performed cellular analysis of B cell differentiation during the primary response. We used a FACS strategy to detect germinal center B cells and memory B cells based on differential expression of CD38 among Ag-binding lineage (−) B220+ cells (15). As shown in Fig. 5A, the frequency of germinal center B cells (lineage [−] CD38Δull−/−NP+ B220+ cells) in IA-B mice was reduced compared with that in WT mice at 1 wk postimmunization, but it recovered to the level of WT mice due to clonal expansion of <5% of MHC-II–sufficient B cells, as was previously reported (15). The frequency of germinal center B cells in IA-B/CD40LBTg mice was similar to that in IA-B mice at 1 wk postimmunization, but it was greatly reduced after 2 wk postimmunization, being barely detectable at 3 wk postimmunization. Notably, the majority of NP-specific germinal center B cells in IA-B/CD40LBTg mice expressed MHC-II (Fig. 5B), although MHC-II+ cells are <5% of all B cells. Collectively, these results indicate that autonomous CD40/CD40L signaling on B cells is not sufficient for germinal center differentiation. In fact, the frequency of NP-specific germinal center B cells in IA-B/CD40LBTg mice was reduced after 2 wk postimmunization compared with that in WT mice. Therefore, autonomous CD40/CD40L signaling on B cells appeared to terminate ongoing germinal center response.
Reduced size and quality of IgG memory B cell compartment in IA-B/CD40LTg and CD40LTg mice

During the early primary response, similar frequencies of B cells with memory phenotype (lineage [−]CD38<sup>hi</sup>/NP<sup>+</sup>B220<sup>+</sup> cells) were detected in WT mice and CD40LTg mice, although the frequency of memory B cells in IA-B/CD40LTg mice was reduced compared with IA-B mice at 3 wk postimmunization (data not shown), possibly as a result of early termination of germinal center B cell differentiation. We further examined the size of the IgG memory B cell compartment in the late primary response at 20 wk postimmunization. Because CD40LTg and IA-B/CD40LTg mice have enlarged spleens compared with controls, the absolute numbers of total IgG<sup>+</sup> B cells and NP-binding IgG<sup>+</sup> B cells in spleen were compared between their controls (cd19cre<sup>−/−</sup>). As shown in Fig. 6A, the numbers of NP-binding IgG<sup>+</sup> B cells in CD40LTg mice and IA-B/CD40LTg mice were significantly reduced compared with their counterparts, whereas the total numbers of IgG<sup>+</sup> B cells were similar compared with controls. In addition, the numbers of total IgG<sup>+</sup> and anti-NP IgG<sup>+</sup> Ab-secreting cells in the bone marrow of CD40LTg mice were reduced compared with those in their control mice (Fig. 6B). Anti-NP2 and -NP15 IgG<sup>+</sup> Ab-secreting cells were barely detectable in IA-B/CD40LTg mice (data not shown). Consistent with the serum-affinity maturation data (Fig. 4D), the ratio of high-affinity (captured by NP2-BSA) versus total (captured by NP15-BSA) anti-NP IgG<sup>+</sup> Ab-secreting cells in the bone marrow of CD40LTg mice was significantly reduced ($p = 0.03$) compared with that in control mice (Fig. 6C). Collectively, these results indicate that expression of CD40L on B cells reduces the size and the quality of the Ag-specific IgG memory compartment.

CD40L expression on B cells did not allow MHC-II–independent germinal center differentiation but resulted in reduced numbers of MHC-II–dependent germinal center B cells in association with enhanced IgG<sub>1</sub> Ab production

We performed adoptive-transfer studies to test whether the effect of the CD40L expression was due to intrinsic autonomous CD40/CD40L signaling on B cell differentiation. MHC-II–sufficient or -deficient MZ B cells and FO B cells were purified from IA-B mice, IA-B/CD40LTg mice, or control (cd19cre<sup>−/−</sup>) mice and were transferred into iab neo/neo cd19<sup>cre/cre</sup> recipient mice. We used iab neo/neo cd19<sup>cre/cre</sup> mice as recipients because they cannot generate T cell-dependent germinal centers as a result of B cell-specific MHC-II and CD19 double deficiency. One day after adoptive transfer, recipient mice were immunized with NP-CGG plus alum, and the frequency of donor-derived Ag-specific germinal center B cells was analyzed by FACS. As shown in Fig. 7A for day 7 after immunization, iab neo/neo cd19<sup>cre/cre</sup> mice that received MHC-II–sufficient (i.e., MHC-II<sup>−/−</sup>CD40LTg<sup>−/−</sup>) MZ B cells or FO B cells generated NP-binding CD38<sup>hi</sup>/PNA<sup>+</sup> germinal center B cells, as expected. However, iab neo/neo cd19<sup>cre/cre</sup> mice that received MHC-II–deficient (i.e., MHC-II<sup>−/−</sup>CD40LTg<sup>−/−</sup>) MZ or FO B cells or MHC-II–deficient CD40L-expressing (i.e., MHC-II<sup>−/−</sup>CD40LTg<sup>−/−</sup>) MZ or FO B cells barely generated B cells.
with germinal center phenotype by day 7 (Fig. 7A, 7B) or by day 14 after immunization (data not shown). As expected, NP-binding germinal center B cells were not detected in immunized control iab neo/neo cd19 cre/cre mice without B cell transfer. Therefore, we concluded that CD40L expression on B cells was not sufficient to induce germinal center differentiation of MHC-II-deficient B cells in immunized mice and that signals brought about through MHC-II-restricted Ag presentation are absolutely essential for germinal center differentiation of MZ B cells and FO B cells in response to T cell-dependent Ag.

We next examined the effect of CD40L expression on germinal center differentiation by MHC-II-sufficient B cells. When we transferred MHC-II-sufficient CD40LTg B cells into iab neo/neo cd19 cre/cre mice, recipient mice developed diarrhea and significant weight loss (data not shown). Therefore, C57BL/6 CD45.1 mice were used as recipients in this experiment. To normalize the basal affinity of B cell AgR of B cells from control and CD40LBTg mice, B cells from anti-NP Ig H chain Tg mice (QM mice) (18) on a C57BL/6 CD45.2 background, with or without B cell-specific CD40L expression, were used as donor B cells. Thus, an anti-NP
Ig H chain transgene (Igha haplotype) was introduced onto each donor mouse background (i.e., WT or CD40LBTg) by interbreeding with QM mice. The frequency of NP-binding B cells determined by FACS was ~3% of total B220+ B cells in QM mice, regardless of CD40LBTg expression. As shown in Fig. 7C and 7D for day 7 analysis after immunization with NP-CGG plus alum, the frequency of NP-binding donor-derived CD45.2+ cells (R2) in CD45.1 mice that received MZ and FO B cells of CD40LTg QM mice (CD40L-QM) (0.4 and 0.4%, respectively) were lower than the frequency of CD45.2+ cells in the recipient mice that received MZ and FO B cells of control QM mice (WT-QM) (1.7 and 0.9%, respectively). The majority of donor-derived NP-binding CD45.2+ B cells (R2) were CD95+CD38−, the phenotype of germinal center B cells.

After immunization with T cell-dependent Ag NP-CGG adsorbed to alum, extrafollicular plasma cell differentiation of Ag-specific B cells proceeds at around day 5 before germinal center differentiation at around day 7, with the latter resulting in high-affinity IgG1 plasma cell production (21). We next examined the magnitude of donor QM B cell-derived NP-specific IgM+ and IgG+ Ab production by ELISA at 5 and 7 d after adoptive transfer of WT-QM or CD40LTg-QM B cells into CD45.1 recipient mice, followed immediately by immunization with NP-CGG adsorbed to alum. The frequencies of donor-derived germinal centers in mice that received CD40LTg B cells were equivalent to or less than those in controls at day 5 and 7 of immunization (Fig. 7E). As shown in Fig. 7F; anti-NP-specific IgM+ and IgG+ titters were similar between these two groups at day 5. However, the group that received CD40LTg B cells produced significantly higher anti-NP IgG+ titters compared with those that received WT B cells at day 7 of immunization.

**Massive production and accumulation of Ag-specific plasma cells in T cell areas in association with germinal center response in CD40LTg mice**

We next conducted immunofluorescent histology to visualize the process of Ag-specific germinal center formation and plasma cell production in CD40LTg mice in the early primary response. Three serial sections from immunized mouse spleens were stained with a combination of anti-IgD Ab, NP-PE, anti-CD138 Ab, and/or anti-TCR Ab to reveal the localization of Ag-specific NP-PE-binding CD138+ plasma cells. Germinal centers were identified as IgD+TCR− areas, surrounded by IgD− naive FO B cells. Small clusters of bright NP-PE–staining cells (i.e., NP-specific extrafollicular plasma cells) appeared by day 4.5 postimmunization in bridging channel regions in spleens of immunized WT control mice, whereas bright NP-PE–staining cells were somewhat less frequent in the spleens of CD40LTg mice and were scattered randomly in the red pulp (data not shown). By day 7, as shown in Fig. 8A, many clusters of NP-PE–bright-staining plasma cells were found in the red pulp outside of IgD+ B cell follicles of WT spleen. Also, some Ag-specific germinal center cells were recognized as NP-PE–dull IgD− areas (indicated by arrowheads) adjacent to TCR+ T cell areas. These Ag-specific germinal centers in WT mouse spleen showed increased intensity of NP-PE staining from days 7–21, indicating advanced affinity maturation. At day 7 postimmunization in CD40LTg mice, NP-PE–bright-staining plasma cells accumulated in T cell areas adjacent to NP-specific germinal centers (arrowheads, Fig. 8A). However, by day 14, germinal center structures were no longer clear in CD40LTg spleen; instead, the T cell areas were completely filled with large numbers of NP-specific plasma cells. By day 21 postimmunization, large clusters of NP-PE+ plasma cells were no longer found in T cell areas; however, the few NP-PE+ plasma cells remaining at this point were exclusively found in T cell areas.

In contrast, NP-PE–staining cells were barely detectable in IA-B mice before day 7 postimmunization (data not shown), because, as previously reported (15), extrafollicular plasma cell differentiation and germinal center response in these mice were significantly impaired as the result of B cell-restricted MHC-II deficiency. NP-PE staining was also found occasionally in the red pulp of IA-B/CD40LTg mice at day 7 postimmunization (data not shown). As shown in Fig. 8B, at day 14, NP-specific germinal centers that were recognized as NP-PE–dull IgD− areas (indicated by arrows) were evident in IA-B mice and were also occasionally seen in IA-B/CD40LTg mice. As was expected from the results in Figs. 5B and 7A, these NP-specific germinal centers in IA-B and IA-B/CD40LTg mice expressed MHC-II (data not shown). In addition, bright NP-PE–staining cells (i.e., NP-specific plasma cells) and CD138+ plasma cells were detected in the red pulp area, but not in the T cell areas, of IA-B or IA-B/CD40LTg mice.

Because NP-specific plasma cells in CD40LTg spleen emerged in T cell areas adjacent to Ag-specific germinal centers but not in the red pulp after day 7 postimmunization, they are not typical extrafollicular plasma cells that are produced prior to germinal center response during the first week of primary response. Also, the accumulation of NP-specific plasma cells was not apparent in the T cell areas of IA-B/CD40LTg mice that largely lack MHC-II–dependent germinal center response at days 7 and 14 postimmunization. Furthermore, the emergence of Ag-specific plasma cells in T cell zones was associated with the reduction of germinal center B cells in CD40LTg mice. Therefore, it is anticipated that

**FIGURE 8.** Ag-specific germinal center and plasma cell differentiation in CD40LTg and IA-B/CD40LTg mice. A. Three serial spleen sections from WT mice and CD40LTg mice at days 7, 14, and 21 postimmunization with NP-CGG plus alum were stained with biotinylated anti-IgD Ab plus streptavidin-Alexa 488 plus NP-PE, CD138-PE, or anti-TCR6-PE. Serial sections are arranged in a row. Arrowheads indicate NP-specific germinal centers. B. Three serial spleen sections from IA-B mice and IA-B/CD40LTg mice at day 14 postimmunization with NP-CGG plus alum were stained as in A. Arrowheads indicate NP-specific germinal centers. Original magnification ×100.
NP-specific plasma cells in T cell areas of CD40LBTg spleen are plasma cells directly produced from adjacent NP-specific germinal centers. These results support the notion that augmented early primary response in CD40LBTg mice is due to robust Ag-specific plasma cell differentiation from prematurely terminating germinal centers.

**Discussion**

CD40/CD40L interaction may be one of the most well-studied topics in immunology. However, it is still not entirely clear how different providers and the timing and duration of CD40/CD40L signal transduction affect B cell differentiation during humoral response. In this study, we generated a new mouse model (i.e., IA-B/CD40LBTg mice), in which B cells express CD40L under the IgVH promoter but lack MHC-II, to determine the role of CD40 signaling on B cells in the absence of MHC-II–mediated cognate T cell help. Using these mouse models, we showed that B cell autonomous CD40 signaling significantly enhanced Ab production in response to T-independent and -dependent Ags. However, autonomous CD40 signal was not sufficient for the induction of germinal center B cell differentiation in the absence of MHC-II–restricted cognate T cell help; in fact, impaired MHC-II–dependent clonal expansion of Ag-specific B cells and terminated ongoing germinal center response. Furthermore, this resulted in the production of large numbers of Ag-specific plasma cells that, unusually and temporarily, accumulated in T cell areas.

Although the mechanisms of action of autonomous CD40/CD40L signaling on B cells is not entirely clear, CD40L expressed on B cells stimulates them via an autocrine pathway, without stimulation of dendritic cells (22). Furthermore, as shown in this study, in vitro anti-CD40 Ab treatment triggered strong NF-κB activation in CD40LBTg B cells (Fig. 1). In this context, CD40LBTg mice are different from recently reported latent membrane protein 1 (LMP1)/CD40 mice, which express a constitutively active CD40R in the form of a LMP1/CD40 chimeric protein (14). The latter mice show spontaneously activated B cell phenotype in association with constitutive activation of the noncanonical NF-κB pathway (14). However, CD40LBTg mice also had increased numbers of mature B cells, in association with splenomegaly-like LMP1/CD40 mice, and exhibited differential gene expression levels of *prdm1* and *irf4* in MZ B cells (Fig. 2). Therefore, it is possible that noncanonical NF-κB activation in CD40LBTg mice is elevated during B cell development or B cell differentiation in vivo, which may impact humoral response in CD40LBTg mice.

Previous studies explored the requirement of CD40/CD40L engagement during germinal center B cell differentiation; however, the requirement of CD40 signaling is still controversial. Consistent with the observations from CD40- or CD40L-deficient mice, in vivo anti-CD40 Ab treatment completely blocked germinal center formation (23, 24) or disrupted established germinal centers (23) and impaired memory response (24). However, in vivo blockade of CD40/CD40L interaction by soluble CD40-γ1 fusion protein had no effect on germinal center differentiation, but it led to a strong reduction in memory B cell generation (25). CD40-agonistic Ab treatment could not completely convert model T cell–independent germinal centers, which were induced by strong cross-linking of BCR (26), into a T cell–dependent germinal center phenotype and failed to generate memory response (27). Also, in vitro studies showed that CD40 and B cell AgR dual triggering of human resting B cells turned on a partial, but not complete, germinal center phenotype (28). Furthermore, agonistic anti-CD28 Ab treatment generated virus Ag-specific germinal centers in the complete absence of CD40L (29), suggesting that CD40 signaling on B cells is not required for their differentiation into germinal center B cells. Finally, DNA microarray analysis indicated that germinal center expansion occurs in the absence of CD40 signaling (30), except there was significant evidence for CD40 signaling in the differentiation of centrocytes into memory B cells and plasma cells (30). In this context, our current study showed that CD4* T cell–independent B cell–autonomous CD40/CD40L signal could not induce germinal center B cell differentiation in the absence of MHC-II–dependent cognate T–B interaction. Thus, our results support the notion that other signals derived from CD4* T cells during cognate MHC-II–restricted interaction are critical to B cell differentiation into germinal center phenotype.

To our surprise, constitutive CD40L expression on B cells terminated ongoing germinal center B cell differentiation at an early stage of immune response (i.e., 1 wk postimmunization) in IA-B/CD40LBTg mice and also in CD40LBTg mice compared with their controls (Fig. 5A). The effect was severe in IA-B/CD40LBTg mice, in which the recovery of germinal center formation is absolutely dependent on extensive clonal expansion of the <5% of B cells that are still MHC-II sufficient (15). Recent studies established that ICOS-L/ICOS engagement upon interaction between Ag-specific B cells and FO Th cells is critical for germinal center differentiation of B cells (31, 32). It is also suggested that one of the critical roles of CD40 signal for germinal center B cell differentiation is to upregulate LTeβ expression on B cells (33). In this context, expression of costimulatory molecules, such as ICOS-L, and OX40L, on CD40LTg B cells by FACS and LTβ expression by QRT-PCR were similar to those on control B cells (data not shown). Also, the frequency of CXCR5+ or ICOS+ cells among total CD4+ cells in CD40LBTg mice and control mice at the onset of germinal center generation (day 4) was comparable (data not shown). The early termination of germinal centers is consistent with the work of other investigators (22). Also, in relation to this, Erickson et al. (34) reported that germinal center formation was ablated in mice treated with agonistic anti-CD40 Abs, and LMP1 Tg mouse models mimicking CD40 signaling on B cells failed to develop germinal centers (14, 35). These studies collectively support the notion that strengthened CD40 signaling on B cells reduces the magnitude of germinal center response.

A possible implication for the restrained germinal center response in CD40LBTg mice was that naive CD40LBTg FO B cells were conditioned to take an extrafollicular plasma cell differentiation pathway, rather than the germinal center pathway, as the result of enforced CD40 signaling, as previously suggested in the study by Ericsson et al. (34). Consistent with this notion, CD40LBTg and IA-B/CD40LBTg mice generated augmented early humoral response. However, immunohistology did not reveal enhanced extrafollicular plasma cell differentiation in those mice. It should also be taken into account that enhanced early humoral response found in CD40LBTg mice and IA-B/CD40LBTg mice, compared with their control mice, could simply be due to their enlarged spleen size and increased B cell numbers. Therefore, the previous notion that anti-CD40 signal promotes extrafollicular plasma cell differentiation at the expense of initiation of the germinal center pathway (34) cannot explain our observations. In fact, by adoptive-transfer experiments, CD40LTg B cells produced increased IgG1 compared with control B cells at day 7 but not day 5 (Fig. 7F), with the generation of equivalent or fewer germinal center B cells (Fig. 7E). As evident from histology, plasma cell differentiation in CD40LBTg mice seemed to occur from established germinal centers, which resulted in immediate, but temporal, massive accumulation of plasma cells in T cell areas (Fig. 8A) and augmented Ag-specific IgG1 production in the early primary response (Fig. 4C). Thus, autonomous CD40/CD40L signal does not block the initiation of germinal center differentiation, but it...
prematurely terminates ongoing germinal center response, which leads to Ag-specific IgG1 plasma cell production.

Activation of CD40 leads to NF-κB–mediated induction of the IFN regulatory factor 4 transcription factor, which, in turn, represses bcl-6 expression by binding to its promoter region (36). A greater concentration of IFN regulatory factor 4 induced Prdm1 and consequently induced the transition from a germinal center gene-expression program to that of a plasma cell (37). Prdm1 is the master transcription factor of plasma cell differentiation (38, 39) and is a repressor for various genes, including myc and bcl-6, which are involved in cellular proliferation (38), essential for germinal center formation (40, 41). In this context, freshly isolated MZ B cells from CD40LTg mice expressed an increased level of prdm1 and irf4 compared with non-Tg counterparts (Fig. 2), which is consistent with gene regulation in B cells that received anti-CD40 stimulation. Furthermore, premature termination of germinal center response coincided with temporal production of Ag-specific plasma cells in T cell areas (Fig. 8A). It should be noted that although the absolute numbers were reduced, memory B cells were also generated in CD40LTg mice. Therefore, we propose that our study may provide physiological evidence of CD40 signaling as the active termination signal for germinal center B cells, which was recently suggested by work by Dalla-Favera and colleagues (30, 36, 42). Of course, future studies are needed to further clarify gene regulation of germinal center B cells and additional signaling requirements that delineate plasma cell versus memory B cell differentiation.

Aberrant CD40L expression on B cells has been observed in systemic lupus erythematosus patients (9) and in lupus-prone BXSB mice (10). Indeed, the CD40LTg B cell mouse model proved that aberrant CD40L expression on B cells induces lupus-like symptoms (12). CD40LTg MZ B cells exhibited enhanced proliferation and plasma cell differentiation in vitro (data not shown). Therefore, aberrant CD40L expression on B cells may have a significant impact on lupus by promoting plasma cell differentiation of self-reactive B cells, leading to autoantibody production, without the need for cognate help from autoreactive CD4+ T cells. It is also an open question whether the unusual accumulation of plasma cells in spleen T cell areas in CD40LTg mice might have an impact on the development of autoimmunity in CD40LTg mice. Interestingly, Ab-producing cells in Fas-deficient mice are also dominantly localized in T cell areas (43). In this respect, CD40LTg and IA/B-CD40LTg mice should provide unique tools to further investigate the significance of CD40 signaling in autoimmunity.

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Disclosures

The authors have no financial conflicts of interest.

References


Corrections


The authors revised the grant footnote to correct the third grant number. The corrected grant footnote is shown below.

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