Short-circuiting long-lived humoral immunity by the heightened engagement of CD40

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Received for publication August 30, 2001, and accepted in revised form January 21, 2002.

Agonistic αCD40 Ab’s have been shown to be potent immune adjuvants for both cell- and humoral-mediated immunity. While enhancing short-lived humoral immunity, the administration of a CD40 agonist during thymus-dependent immune responses ablates germinal center formation, prematurely terminates the humoral immune response, blocks the generation of B cell memory, and prevents the generation of long-lived bone marrow plasma cells. Interestingly, some of these effects of heightened CD40 engagement could be mimicked by enhancing the magnitude of antigen-specific T cell help. Taken together, these studies demonstrate that as the magnitude of CD40 signaling intensifies, the fate of antigen-reactive B cells can be dramatically altered. These are the first studies to describe the multifaceted function of CD40 in determining the fate of antigen-reactive B cells and provide novel insights into how CD40 agonists can short-circuit humoral immunity.


Introduction
Interrupting CD154-CD40 interactions prevents the development of thymus-dependent (TD) humoral responses and many cell-mediated immune responses (1). Conversely, it has been shown that agonistic αCD40 Ab’s administered in vivo can act as potent immune adjuvants for both arms of the immune response. Several reports have shown that αCD40 enhances the immune response to thymus-independent (TI) antigens (2, 3) and can amplify cell-mediated immune responses to viruses and tumors (4–6). Given these observations, the use of CD40 agonists to boost the immune response of the immunocompromised host has been considered.

We have investigated whether CD40 agonists can influence the follicular versus extrafollicular differentiation of antigen-experienced B cells. Antigen-specific B cells that are activated by CD154-dependent cognate interactions with T cells traffic either to follicles or to extrafollicular sites. Follicular B cells in the spleen that are recruited to form germinal centers (GCs) ultimately differentiate to memory B cells and/or to long-lived plasma cells that migrate to the bone marrow where they sustain high-affinity, antigen-specific Ig levels in the serum (7–11). In contrast, B cells that emigrate to extrafollicular foci within the red pulp, following interactions with T cells, locally differentiate to short-lived Ab-secreting cells (ASCs) that produce low-affinity Ig (12–14). Here, we document that the administration of a CD40 agonist (αCD40 mAb), in combination with a TD antigen, ablates GC formation and induces a pattern of extrafollicular B cell differentiation in the spleen and lymph node. This CD40-induced deviation resulted in a heightened early humoral immune response, the premature termination of the humoral immunity, loss of long-lived bone marrow plasma cells, and memory B cell development. Interestingly, priming of the T cell compartment to enhance CD40 signaling mimicked some but not all of the effects of the αCD40 agonist. This latter data indicates that extrafollicular B cell differentiation by heightened CD40 signaling may represent a physiological means to limit the duration and intensity of the humoral immune response. Taken together, these studies demonstrate that αCD40, as well as the magnitude of helper T cell activity, can influence the follicular/extrafollicular fate of antigen-reactive B cells.

Methods
Mice. C57/BL6 Ly5.2 congenic mice and CD40−/− C57/BL6 mice, 6–8 weeks old, were bred and maintained in the specific pathogen-free animal facility at Dartmouth Medical School, Lebanon, New Hampshire, USA. QM mice were backbred to the C57/BL6 JH−/− Jk−/− strain for nine generations to produce QM F9 C57/BL6 mice. IghCD40 mice were generated as described previously (15). Briefly, wild-type human CD40 cDNA was cloned into the pEmVhp expression vector containing the mouse rearranged H chain promoter derived from...
pMO-μ4C8 and microinjected into C57/BL6 oocytes. Mice were backcrossed to murine CD40+/– mice of a C57/BL6 background, thereby allowing the expression of human CD40 exclusively in the B cell compartment.

**B cell preparation and adoptive transfer.** Spleen cell suspensions isolated from QM mice were treated with ammonium chloride-Tris buffer and were T cell depleted using anti-Thy-1.2 plus anti-CD4 Ab and rabbit complement (Accurate Chemical & Scientific Corp., Westbury, New York, USA). An aliquot of cells was stained with the R2348.8 4-hydroxy-5-nitrophenylacetlyl (NP) idiotype-specific mAb and the pan B cell marker, B220, and analyzed by flow cytometry (FACScan; Becton Dickinson Immunocytometry Systems, San Diego, California, USA) to determine percentage of Tg B cells. In all experiments, normal rat serum was added to minimize nonspecific staining. Tg B cells (3 × 10⁶) were adoptively transferred intravenously into sex-matched C57/BL6 Ly5.2 recipient mice. All experiments consisted of three mice per group.

**Immunization and CD40 treatment.** NP30-KLH and NP-Ficoll were purchased from Solid Phase Sciences Inc. (San Rafael, California, USA). To examine TD responses, recipient mice were challenged intraperitoneally 24 hours after adoptive transfer with CFA alone or with 100 µg NP-KLH emulsified in CFA in the absence or presence of FGK115. FGK115, an agonistic rat IgG2a anti-mouse CD40, was HPLC-purified and administered at 100 µg intraperitoneally on day 0, 2, and 4 after immunization. As indicated, control rat IgG was administered similarly to FGK115. In some experiments, mice were immunized intraperitoneally with 200 µl of a 10% solution of sheep blood alsevers (Colorado Serum Co., Denver, Colorado, USA) and treated with S2C6 (16), an agonistic anti-human CD40 mAb. Mice were killed on day 7, and intact spleens were removed and sera collected by retro-orbital eye bleeds. For experiments using carrier-primed T cells, recipient mice were challenged intraperitoneally with 100 µg KLH emulsified in CFA 1 week prior to cell transfer. Recipients were immunized intraperitoneally and subcutaneously with 100 µg NP-KLH precipitated in alum, and analyses were performed on day 5 after immunization. Secondary immune responses were assessed by pooling T cell–depleted splenic B cells from primary recipients 30 days after immunization and by transferring 10 × 10⁶ cells intravenously into secondary recipients that were KLH primed 2 weeks previously. Hosts were challenged with alum alone or with 100 µg NP-KLH alum 24 hours after transfer, and serum was collected 7 days later. Endogenous levels of high-affinity anti-NP IgG1 and IgG2a were detected by ELISA. Long-lived bone marrow ASCs generated from the spleen or lymph node were quantified by ELISPOT 7 days after transferring normalized numbers of Tg B cells into naive secondary hosts.

**Flow cytometric reagents.** The following mAbs were used for multicolor flow-cytometric analysis: 6B2, a rat IgG1; anti-mouse B220, a rat IgM anti-mouse GL7; and CD45.2, a rat IgG2a, anti-mouse Ly5.1 and Ly5.2, were purchased from PharMingen (San Diego, California, USA). FITC-conjugated Ab90, a rat IgG2a anti-mouse CD38, and FITC-conjugated peanut agglutinin (PNA) were purchased from Sigma Chemical Co. (St. Louis, Missouri, USA). The anti-VH17.2.25 cell line (R2348.8) was generously provided by Theresa Imanishi-Kari (University of California San Francisco, San Francisco, California, USA). Streptavidin-phycocerythrin (streptavidin-PE) and FITC were obtained from Southern Biotechnologies Associates (Birmingham, Alabama, USA). For removal of cytophilic Ig, cells were washed in 5% FCS in BSS and resuspended in low-pH (pH 4.0) acetate buffer containing 0.05 M Na acetate, 0.085 M NaCl, and 0.005 M KCl and 2% FCS in distilled H₂O. Samples were incubated on ice for 1 minute followed by the addition of an equal volume of 0.1 M Tris buffer, pH 8.0, containing 2% FCS. Samples were washed twice in 5% FCS BSS and then stained. Chromatographically purified rat IgG was used as isotype control. A minimum of 200,000 events per sample were collected on a FACSScan, and dead cells were excluded based on forward and 90° light scatter.

**ELISA and ELISPOT reagents.** NP-25–BSA or NP-4–BSA was used to capture NP-specific Ab’s in a sandwich-based ELISA. Allotypic-specific (Igha or Ighb) goat anti-mouse IgG1 and IgG2a conjugated to alkaline phosphatase (Southern Biotechnology Associates) were used to detect Ab derived from Tg or endogenous B cells with subsequent development with phosphatase substrate (Sigma Chemical Co.). Absorbance was read at a dual wavelength of 405 and 540 nm using an MR700 plate reader (Dynatech Laboratories, Chantilly, Virginia, USA). Serum from a hyperimmunized QM mouse was used as a standard in all assays to determine nanograms per milliliter. For ELISPOT analysis, a modified manufacturer’s protocol was used from Millipore Corp. (Beverly, Massachusetts, USA). Briefly, Millipore MultiScreen HA plates were coated with either NP-25–BSA or NP-4–BSA. Normalized numbers of Tg B cells were plated in triplicate at a starting concentration of 2.4 × 10⁶ cells, serially titrated, and incubated at 37°C for 5 hours. Tg ASCs were detected with allotype-specific (Ighb) biotin-conjugated rat anti-mouse IgG1 followed by development with streptavidin–horse radish peroxidase and chromogen substrate, and counted.

**Confocal image analysis.** On day 5 or 7 after immunization, intact spleens and lymph nodes were harvested and 4- to 5-µm frozen sections were fixed in acetone at 4°C for 15 minutes and allowed to air dry at 4°C for 48 hours prior to staining. Sections were stained in 1% BSA PBS using a three-color protocol with rat anti-mouse Ab’s: 6B2 FITC, CD4+, and CD8-PE, and Cy5-conjugated anti-NP idiotype Vh17.2.25. To block nonspecific binding, 1% mouse serum and 10% normal rat serum were included in the staining process. Samples were analyzed on a Bio-Rad 1024 confocal microscope (Bio-Rad Laboratories Inc., Hercules, California, USA). RT-PCR. On day 7 after immunization, T cell–depleted splenocytes were dual stained with biotin-conjugat-
ed anti-NP idiotype VH17.2.25 and Ly5.1 to label Tg B cells. Tg B cells were positively selected (≥ 90% purity) using streptavidin-coupled microbeads (Miltenyi Biotec, Auburn, California, USA). Total RNA (1 µg each) isolated from purified Tg B cells by using RNeasy system (QIAGEN Inc., Valencia, California, USA) was used as a template for cDNA synthesis with Superscript II System (Life Technologies Inc., Rockville, Maryland, USA). PCR amplification was carried out by titrating cDNA samples with Taq (Boehringer Mannheim GmbH, Mannheim, Germany) and the primers for HPRT to normalize levels of template. Amplification of BSAP and Blimp-1 transcripts were carried out using normalized amounts of cDNA. The primers used were as follows: 5′-GCTGGTGAAGGCCTCT-3′ (HPRT forward) and 5′-CACAGGACTAAGACCTGC-3′ (HPRT reverse); 5′-TCCTCGGACCACGAGCAG-3′ (BSAP forward) and 5′-CCTGGTGGATGGAGACGC-3′ (BSAP reverse); 5′-TCCGCTCCGTAAGTTCA-3′ (Blimp-1 forward) and 5′-GGTGAAACTCTCCTCTGGAAT-3′ (Blimp-1 reverse). Conditions used for amplification were 94°C, 55°C/65°C, and 72°C for 35 cycles. HPRT was used as a control to confirm the amount of each template.

Results

Anti-(α)CD40 administration aborts GC B cell differentiation. We have developed a system using Tg B cells that faithfully recapitulates the humoral immune response to TD antigens and provides the means to track clonal B cell behavior in vivo. Tg B cells can be clearly identified by virtue of their antigen specificity to NP, the expression of the VH17.2.25 idiotype, and by the expression of the allotypic marker, Ly5.1, when using congenic Ly5.2 mice as recipients. Tg B cells vigorously expand by day 7 after challenge with the TD antigen, NP-KLH (2). To evaluate how CD40 agonists alter clonal expansion and differentiation of B cells during TD responses, recipients were immunized in the presence or absence of agonistic αCD40 mAb. Total numbers of Tg B cells present in the spleens of immune mice were quantified at day 7 after immunization. Immune Tg B cells expanded approximately threefold over naive control animals, regardless of control Ig or αCD40 treatment (Figure 1a). As demonstrated here and reported previously (2), Tg B cells failed to undergo expansion in recipients that were administered αCD40 in the absence of antigen. These results indicate that Tg B cell expansion is antigen-driven and that increasing CD40 engagement by the addition of αCD40 mAb does not enhance the proliferative phase of Tg B cell activation.

A hallmark of follicular B cell differentiation is the generation of GCs. GC formation and kinetics in this Tg system can be readily quantified by flow cytometric analysis of the Tg B cells using a panel of GC-specific markers (17–19). Tg B cells from the spleens of immune recipients exhibited a GC phenotype as demonstrated by the upregulation of PNA and GL7 staining and the downregulation of CD38 expression (Figure 1b). In con-

Figure 1

αCD40 treatment aborts a GC phenotype and promotes extrafollicular localization of Tg B cells. (a) Total numbers of splenic Tg B cells (anti-NP idiotype+, B220+) were measured on day 7 after immunization. (b) Spleen or LN cells from naive (black line), immune/RIgG (red line), and immune/αCD40 (blue line) were stained with GC markers. Histograms shown represent the relative intensity of the indicated marker on Tg B cells (B220+ Id+). (c) Spleen cells from naive and immune IghCD40 transgenic mice treated with rat IgG or agonistic αhCD40 mAb were stained with GC markers. The percentage of total PNA+ GL7+ GC B cells are indicated in each panel. (d) Frozen sections from intact spleens were costained with B220 (green), CD4, and CD8 (red), and anti-NP idiotype (Id) 17.2.25 (blue) Ab’s, and analyzed by confocal microscopy. Objective: ×20; zoom 1.5. Data are representative of three independent experiments.
CD40 agonists reduce the duration of humoral immune responses and ablate the generation of B cell memory. The dramatic alterations in follicular B cell differentiation induced by CD40 agonists would predict that CD40 agonists should alter the intensity or duration of the overall humoral immune response. Hence, the magnitude and longevity of the serum IgG1α anti-NP (derived from Tg B cells) was measured over a 3-week interval when CD40 was or was not administered. CD40 treatment of immune recipients facilitated an earlier onset of NP-specific IgG1α production and showed twofold greater amounts of IgG1α anti-NP over mice challenged with NP-KLH alone at day 7 after immunization (Figure 2). Furthermore, CD40 treatment altered the kinetics of IgG1α anti-NP responses by caus-
ing the premature termination of responses by 10 days after immunization. Thus, αCD40 administration modestly enhanced early humoral immune responses but terminated the humoral immune response prematurely.

Since it appeared that αCD40 aborted GC formation and prematurely terminated humoral immunity, we assessed whether αCD40 could prevent the generation of NP-specific memory B cells using normal, non-Tg B cells. Mice were immunized with NP-KLH and administered αCD40 or control rat Ig. After 30 days, B cells were isolated, transferred to secondary recipients, and challenged with antigen. Mice that received immune splenic B cells produced significant titers of high-affinity NP-specific IgG1b and IgG2ab upon antigenic challenge (Table 1). In contrast, secondary hosts that were given B cells from mice that were immunized and administered αCD40 failed to generate a memory response when rechallenged with antigen. Thus, αCD40 administration prevents the generation of memory B cells.

αCD40 agonists prevent the generation of long-lived plasma cells in the bone marrow. Previous reports have shown that a significant proportion of splenic Ab-secreting cells (ASCs) that develop from extrafollicular differentiation have a lifespan of less than 3 days (13, 20), thus providing local short-term Ab production. Study of the generation of long-lived plasma cells has revealed that many of these are likewise derived in the spleen, but unlike short-lived ASCs, long-lived plasma cells develop from the follicular pathway and migrate to the bone marrow where they can persist for months (10, 21). Because αCD40 treatment terminates humoral immunity prematurely, we anticipated an impact of αCD40 treatment on the generation of long-lived plasma cells. To examine the production of short- and long-lived ASCs in our studies, we performed ELISPOT analyses of spleen and bone marrow in immune mice treated with αCD40. Results from these studies demonstrated that Tg IgG1a ASCs were present in the spleen of all immune recipients 7 days after challenge (Figure 3a). Analysis of splenic ASCs generated by day 21 demonstrated a reduction in Tg IgG1a ASCs in all recipients compared with day 7 and illustrated the short-lived nature of most of the splenic ASCs identified (Figure 3b). High numbers of long-lived IgG1a ASCs were apparent in the bone marrow of immune mice and the majority of these underwent affinity maturation to produce high-affinity (NP-4–binding) Ab (Figure 3c). The administration of αCD40 caused a complete ablation of long-lived bone marrow Tg plasma cells on all days tested. These results show that the terminal differentiation of follicular B cells to long-lived plasma cells is ablated when CD40 signaling is elevated during TD immune responses and explains the short-lived nature of the humoral immune response.

CD40 agonists alter the patterns of Blimp-1 and BSAP expression. The fact that αCD40 caused a marked diversion in B cell differentiation led us to investigate if alterations in differentiation-specific transcription factor expression could be observed. BSAP (B lymphocyte–specific activation protein) and Blimp-1 (B lymphocyte–induced maturation protein 1) are transcriptional factors that have been shown to be expressed during distinct phases of B cell differentiation. BSAP is expressed on all differentiating B cells, except for cells that have terminally differentiated into short-lived ASCs or plasma cells residing in the bone marrow (22). In contrast, Blimp-1 gene expression is rapidly upregulated in B cells that have terminally differentiated into short-lived ASCs or plasma cells residing in the bone marrow (23, 24). We examined

![Figure 3](image-url)

**Figure 3**
αCD40 administration prevents the generation of bone marrow ASCs. Cells from spleen and bone marrow were isolated from the indicated recipients on day 7 (a) and day 21 (b) after immunization and assayed for total NP-specific IgG1a or high-affinity NP-specific IgG1a (c) by ELISPOT using normalized numbers of Tg B cells. Results shown are representative of three independent experiments.

![Figure 4](image-url)

**Figure 4**
Administration of αCD40 upregulates Blimp-1 gene expression in Tg B cells. PCR amplification of the indicated transcript was performed using normalized amounts of cDNA isolated from purified splenic Tg B cells of recipients challenged with NP-Ficoll (TI immune), NP-KLH (TD immune), or NP-KLH and treated with αCD40 (TD immune + αCD40). Data are representative of three mice per group from two independent experiments.
whether these transcriptional factors were regulated in vivo by CD40 agonists during TD immune responses. Tg B cells from recipients were purified on day 7 to 90% or more by magnetic bead selection using the congenic marker Ly5.1, and mRNA levels of BSAP and Blimp-1 were evaluated. Adequate numbers of Tg B cells could be isolated only at the peak of expansion (day 7) and limited the extent of the analysis. RT-PCR analysis demonstrated that BSAP mRNA was expressed at high levels in Tg B cells isolated from recipients that were challenged with NP-KLH compared with naive Tg cells (Figure 4). In contrast, Blimp-1 mRNA expression was present at low levels in immune Tg cells. The differential expression of these transcription factors support the notion that during TD responses, Tg B cells are actively undergoing follicular differentiation. The low levels of Blimp-1 expression from these follicular B cells is not surprising since terminal differentiation of long-lived plasma cell precursors occurs in the bone marrow. Significantly, BSAP mRNA expression was very low in immune Tg B cells that had been purified from recipients treated with αCD40, but high levels of Blimp-1 gene expression were observed. As an additional control, Tg B cells were also purified from recipients that were challenged with the TI antigen, NP-Ficoll, and tested for mRNA expression levels of BSAP and Blimp-1. B cells that respond to TI antigens do not require cognate interactions with helper T cells for activation and therefore culminate in the early activation and local differentiation of B cells to short-lived ASCs (25). Both BSAP and Blimp-1 expression levels were elevated in TI-immune Tg B cells, as expected. These data demonstrate that elevated levels of CD40 signaling diverts B cells along the extrafollicular differentiation pathway and differentiates locally into short-lived ASCs.

Enhanced T cell help mimics some, but not all, of the effects of CD40 agonists on follicular B cell differentiation. The above findings suggest that enhanced CD40 engagement by αCD40 administration directs antigen-reactive B cells to the extrafollicular differentiation pathway. While this provides valuable insights into how CD40 agonists may alter the humoral immune response, does enhanced CD40 engagement by helper T cells (Th cells) exert similar immunological effects on B cell differentiation? The magnitude of CD40 signaling of B cells by Th cells could be enhanced by the increased frequency of Th cells and density of CD154 on memory Th cells. Priming the T cell compartment with antigen in CFA would both increase the frequency of carrier-reactive Th cells and produce a population of memory Th cells that have higher densities of CD154 (26).

We tested whether T cell priming causes alterations in B cell differentiation by using recipient mice that were hyperimmunized with the carrier, KLH, in CFA. Following the transfer of Tg B cells into the carrier-primed recipients, they were challenged (intraperitoneally and subcutaneously) with alum alone or NP-KLH precipitated in alum. This systemic and regional immunization assured T cell priming both in the spleen and LN. In mice that were carrier-primed, immune Tg B cells in both LN and spleen expanded...
approximately tenfold over naive controls (Figure 5a). These levels of B cell expansion were consistently higher than those observed in recipients that were not KLH-primed (Figure 1a) and demonstrated that carrier-primed T cells provided superior helper activity to naive T cells and that they were present in the spleen and LN. The differentiation of Tg B cells in the LN and spleen from naive and carrier-primed immune recipients were evaluated by staining with the GC markers GL7, CD38, and PNA. Immune Tg B cells from the spleen of carrier-primed recipients profoundly expand, however, they fail to express a GC phenotype (Figure 5b), whereas in non-carrier primed mice, Tg B cells do express GC markers in the spleen (Figure 1b). In contrast to that observed in the spleen, immune Tg B cells taken from LN of identical recipients fully differentiated into follicular GC B cells (GL7+ CD38+ PNA+). That is, in an individual mouse, Tg B cells expanded in both the LN and spleen, but in the spleen they diverted to an extrafollicular pathway and in the LN to a follicular pathway. These results were confirmed by examining the histology of spleen and LN sections from carrier-primed recipients. Similar to the histology of αCD40-treated mice (Figure 1d), extrafollicular localization of splenic Tg B cells was observed (Figure 5c). In contrast, Tg B cells from the LNs of the same primed recipients had entered follicles.

The observation that GC formation was selectively overridden by carrier-primed Th cells in the spleen and not the LN implied that long-lived plasma cells may only be generated in the LN under these prescribed conditions. To test this hypothesis, Tg B cells from primed spleen and LN were passively transferred into recipient mice, and the number of ASCs in the bone marrow of recipient mice was determined. Tg B cells from primed LN gave rise to higher numbers of bone marrow plasma cells (Figure 5d). While we cannot directly attribute these effects to heightened CD154 expression on Th cells, the data suggest that when the immune response intensifies, the spleen assumes the responsibility for the production of short-lived, extrafollicular ASCs, while the LN continues to drive B cells through a follicular GC-type response.

Discussion
The studies presented show that the differentiation pathway of antigen-experienced B cells can be altered by a CD40 agonist or the intensity of T cell help. We demonstrate that the administration of a CD40 agonist induces extrafollicular B cell differentiation resulting in the loss in GC formation, a modest increase in serum Ig levels, the premature termination of the humoral immune response, the ablation of long-lived bone marrow plasma cells, and the loss in the generation of B cell memory. The fate of antigen-reactive B cells can also be altered by heightened T cell help, however, in a more restricted, organ-specific fashion.

There has been much interest as to the role of CD40 in the terminal differentiation of B cells. The results presented herein lead to the conclusion that αCD40 administration in vivo directs extrafollicular B cell differentiation. However, in vitro studies of the role of CD40 agonists on the differentiation of B cells are conflicting. On one hand, two published reports have shown that CD40 signaling in vitro can prevent B cell differentiation to plasma cells (27, 28). Arpin et al. have shown that human GC cells cultured with the cytokines IL-2 and IL-10 and CD154-transfected cells acquire a memory B cell phenotype and that the withdrawal of CD154 resulted in terminal differentiation to ASCs (29). On the other hand, a number of reports have shown CD40 signaling in vitro directly induces B cell differentiation and Ab secretion (30–32). First, in vivo and in vitro approaches are vastly different, and second, the in vitro studies were performed in the absence of antigen. Our own studies show that the impact of CD40 agonists are only observed in the presence of antigen and preclude B cells from entering the follicular pathway.

In agreement with our data showing that heightened levels of CD40 signaling promotes extrafollicular B cell differentiation, a recent study by Uchida et al. has demonstrated that overexpression of a constitutive CD40-like signaling molecule, LMP-1, promotes extrafollicular differentiation (33). Uchida and colleagues demonstrated that antigenic challenge of LMP-1 Tg mice induced extrafollicular B cell responses and failed to initiate GC formation and the production of high-affinity Ab’s. As with the αCD40 agonist, signaling through LMP-1 actively suppresses GC formation of differentiating B cells and fosters extrafollicular differentiation. It is noteworthy that Epstein-Barr virus–transformed B cells detected in lymphoid tissues of infectious mononucleosis patients are found neither in primary follicles nor GCs, but in the extrafollicular areas (34, 35). All of these data are in accord with the hypothesis that the triggering of the CD40 signaling cascade by a CD40 agonist or expression of LMP-1 diverts B cell differentiation.

One potential mechanism for the in vivo effects of αCD40 mAb treatment on B cell differentiation is that the mAb may interfere with B cell migration. Experimental data showing that GC responses were ablated in the spleens of carrier-primed recipients suggest that mAb treatment does not directly alter B cell migration. Of interest is whether the differences in GC responses between spleen and LN of hyperimmune mice are due to differences in chemokine responsiveness. A recent study by Hargreaves and coworkers demonstrated that as splenic B cells differentiate into ASCs they undergo changes in their ability to respond to chemokines (36).

Understanding the impact of CD40 agonists on B cell differentiation in vivo both has practical advantages and provides insights into the physiological function of CD40 in terminal B cell differentiation. With regard to the former, CD40 agonists have received attention as potential adjuvants in vaccine development. The in vivo use of agonistic αCD40 Ab’s has been shown previously to enhance isotype-switched Ab responses to TI antigens such as pneumococcal polysaccharides (3) and to ameliorate collagen-induced arthritis with long-term
αCD40 treatment (37). However, our studies demonstrate that while αCD40 administration can transiently enhance antigen-specific Ig levels, it can also enforce the premature termination of long-lived humoral immunity and B cell memory. These findings raise issues with the use of CD40 agonists for enhancing long-term immunity. From a physiological standpoint, these studies have uncovered a novel effect of CD40 agonism during a normal immune response. In a primary immune response, antigen-specific GC responses were readily observed in both the spleen and LN. However, when the helper T cell compartment was hyperimmune, the response in the spleen was diverted to a non-GC, extrafollicular response, while the LN sustained a GC reaction. More in-depth analysis showed that in a carrier-primed mouse the Tg B cells from the LN, but not the spleen, could develop into bone marrow plasma cells. At this time, we cannot directly attribute the differential effects in the spleen versus LN to heightened CD154 expression on the antigen-reactive Th cells, but it is consistent with data presented using a CD40 agonist. More importantly, these studies suggest that as the immune response matures and T cell help intensifies, the function of the spleen and LN diverge, and each assumes distinct responsibilities for sustaining the humoral immune response. Late in an immune response, we would hypothesize that the spleen acquires the responsibility of producing short-lived ASCs, while the LN produces memory B cells and long-lived ASCs for export to the bone marrow. A more extensive testing of this novel hypothesis is currently underway.

Acknowledgments

The authors would like to thank A.G. Rolink and S. Paulie for generously providing the FGK115 (anti-mouse CD40) and S2CG (anti-human CD40) mAbs, respectively, and T. Imanishi-Kari for the kind gift of the R2348.8 cell line (anti-VH17.2.25). The C57/BL6 JH+/+ and Jk−/− mice were kindly supplied by D. Nemazee. This work was supported by grants from the NIH to R.J. Noelle (AI-26296 and AI-42234) and L.D. Erickson (AI-10302-01A1).