gp39–CD40 Interactions Are Essential for Germinal Center Formation and the Development of B Cell Memory

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Summary

gp39, the ligand for CD40 expressed on activated $CD4^+$ T helper cells, is required for the generation of antibody responses to T-dependent (TD) antigens. Treatment of mice with anti-gp39 in vivo inhibits both primary and secondary antibody formation to TD, but not T-independent antigens. However, the role of this receptor-ligand pair in the development of germinal centers and the generation of B cell memory is as yet undefined. Using an antibody to gp39, this study examines the in vivo requirement for gp39–CD40 interactions in the induction of germinal center formation, as well as in the generation of B cell memory. Animals were immunized, treated in vivo with anti-gp39, and evaluated using immunohistochemical staining for the presence of splenic germinal centers 9–11 d after immunization. The results demonstrate that the formation of germinal centers was completely inhibited as a result of treatment with anti-gp39. Moreover, adoptive transfer experiments demonstrate that the generation of antigen-specific memory B cells is also inhibited as a consequence of blocking gp39–CD40 interactions. Taken together, the data demonstrate that gp39–CD40 interactions are critical not only for the generation of antibody responses, but also in the development of B cell memory.

Evidence from a number of studies demonstrate that the interaction between gp39, a molecule expressed on activated Th cells, and CD40, its mitogenic receptor on B lymphocytes, provides the essential signal for the induction of B cell activation and Ig production. In vitro studies have shown that a mAb specific for gp39 (1) and soluble CD40-Ig (2, 3) block the capacity of gp39-bearing Th cells to activate B cells. Moreover, it has recently been demonstrated that in vivo administration of anti-gp39 inhibited both primary and secondary humoral immune responses to a variety of thymus-dependent (TD)[1] antigens (4, 5). The anatomical site at which gp39 expression is most evident is in the periphery of the periarteriolar lymphoid sheath (PALS), where it has been shown to be expressed on $CD4^+$ T cells as a consequence of antigen administration. Furthermore, the gp39-expressing T cells have been shown to be juxtaposed to the B cells producing Ab specific to the immunizing antigen (5). Further evidence that gp39–CD40 interactions are essential for the generation of humoral responses comes from genetic studies of patients with hyper-IgM syndrome (HIM), an immunodeficiency characterized by the inability to respond to TD antigens (for a review see reference 6). A number of studies have demonstrated that the failure of these patients to mount humoral responses to TD antigens can be attributed to mutations in the gene encoding gp39, confirming the requirement for this molecule in Ab-mediated immunity to TD antigens (7–11).

Although it has been established that gp39–CD40 interactions are essential for the development of both primary and secondary humoral responses, the role of this receptor-ligand pair in the development of germinal centers and the generation of B cell memory is as yet unknown. Germinal centers are histologically distinct regions of the spleen consisting of clusters of actively proliferating B cells that develop transiently within the primary follicles of secondary lymphoid organs as a consequence of antigenic stimulation. It has been demonstrated that B cells within a germinal center undergo antigen-driven oligoclonal proliferation (12, 13). Germinal centers are also believed to be the anatomical sites in which isotype switching and affinity maturation occur (14–18). In addition, antigen-specific memory B cell clones (19–21) and plasma cell precursors are thought to be generated within the germinal center (22).

It has been established that T cells are critically important

[1] *Abbreviations used in this paper:* HIg, hamster Ig; HIM, hyper-IgM; PNA, peanut agglutinin; TD, thymus dependent.

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for the formation of germinal centers (23–26), yet the signals required to drive proliferation, differentiation, and memory B cell formation within the germinal center microenvironment have yet to be elucidated. However, some evidence exists which suggests that gp39–CD40 interactions may play a role. Patients with HIM syndrome have no detectable germinal centers and lack B cell memory (6), suggesting that gp39 may be required for germinal center formation and the generation of memory B cells within them. Indirect in vitro evidence that gp39–CD40 interactions may play a role in the germinal center reaction is provided by studies demonstrating that Ab-mediated ligation of CD40 prevents germinal center cells from undergoing apoptosis, a suggested mechanism for the establishment of a memory B cell pool (27, 28). Employing a mAb specific for gp39, this study examines the in vivo requirement for gp39–CD40 interactions in the formation of germinal centers and in the generation of B cell memory. Immunohistochemical analysis of splenic sections from immune animals revealed that in vivo administration of anti-gp39 prevented the formation of germinal centers as detected by both peanut agglutinin (PNA) and membrane IgD (mδ) staining. Moreover, adoptive transfer of splenic B cells from anti-gp39–treated mice demonstrated a lack of antigen-specific memory B cells in the treated animals. Taken together, these data demonstrate that gp39-CD40 interactions are not only critical in the generation of humoral responses, but also in germinal center formation and the generation of B cell memory.

Materials and Methods

Animals. Female, 6–8-wk-old BALB/c and CB17 (BALB/c Igh^b congenic) mice (The Jackson Laboratory, Bar Harbor, ME) were used for the in vivo experiments presented in this study. Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School.

Reagents and Abs. SRBC were purchased from Colorado Serum Co. (Denver, CO). KLH (from *Megathura crenulata*) was purchased from Pacific Marine Biolabs (Inglewood, CA). TNP-KLH and TNP-BSA were prepared as previously described (29). CFA for immunizations was obtained from Sigma Chemical Co. (St. Louis, MO). MR1, hamster anti–murine gp39 mAb (1) was purified by DEAE HPLC from ascites fluid. Hamster Ig (HIg), used as a control Ab, was purified similarly from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY). 10.4.22, a rat anti–murine IgD, was kindly supplied by Dr. Thomas Waldschmidt (University of Iowa, Iowa City, IA) (30). 412-49.7-20, a mAb mouse anti–murine IgG1^b, was a kind gift of Dr. Fred Finkelman (Uniformed Services University of the Health Sciences, Bethesda, MD) (31). PNA-biotin, avidin-horseradish peroxidase (HRP), and 3,3 diaminobenzidine tetrahydrochloride (DAB) were purchased from Sigma Chemical Co.

Anti-gp39 Treatment. HPLC-purified anti-gp39 (MR1) or HIg (as an Ab control) was administered (250 μg/injection, i.p.) on days 0, 2, and 4 after immunization or as indicated for each experiment.

Immunizations for the Generation of Primary Ab Responses. For the generation of primary anti–sheep erythrocyte responses and induction of germinal center formation, mice were immunized intraperitoneally with 100 μl of a 10% solution of SRBC. Animals were killed on day 9 or 11 and immunohistochemistry was performed to detect the presence of germinal centers.

Immunohistochemistry. Splenic tissue was obtained on day 9 or 11 after immunization, snap-frozen in liquid nitrogen, and stored at −80°C until use. 8-μm sections were cut, stored overnight in a humified atmosphere, and fixed for 10 min the following day in fresh acetone containing 0.02% H_2O_2. Sections were then air-dried for 10 min and incubated with either PNA-biotin or anti-IgD-biotin in PBS/0.1% BSA for 1 h at room temperature. After washing three times with PBS, sections were incubated with avidin-HRP for an additional hour at room temperature. Sections were washed and subsequently stained with DAB in 0.05 M Tris-HCl/H_2O_2, pH 7.6, for 10 min. After washing, sections were counterstained with hematoxylin and embedded with Glycergel (Dako Corp., Carpinteria, CA).

Adoptive Transfer of Memory B Cells. CB17 (Igh^b) mice were immunized with 100 μg TNP-BSA (CFA) to induce formation of memory B cells. 4 wk later, B cells from these animals were adoptively transferred into irradiated (600 rad) KLH-primed (100 μg in CFA, i.p.) BALB/c recipient mice. Mice were challenged with 10 μg soluble TNP-KLH at the time of transfer. On day 7, serum was obtained and serum anti-TNP IgG1^b Ab levels assessed using a TNP-specific ELISA.

Antigen-specific Allotype-specific ELISAs. A TNP-specific ELISA was used to determine the IgG1^b anti-TNP titers. Briefly, antigen (1 mg/ml TNP_6-OVA) was absorbed onto flexible polyvinyl microtiter dishes, overnight at 4°C. Plates were washed and blocked with PBS-1% FCS-sodium-azide. Diluted serum samples were incubated for 2 h at 37°C. Samples were washed and the TNP-specific IgG1^b Ab titers determined with biotin-conjugated anti-IgG1^b, followed by avidin-alkaline-phosphatase (Zymed Laboratories, Inc., South San Francisco, CA) as detection. ELISAs were developed by reaction of alkaline-phosphatase with phosphatase substrate (Sigma Chemical Co.). Plates were analyzed on an ELISA reader (model MR700; Dynatech Laboratories Inc., Chantilly, VA) at 410 nm. For antigen-specific ELISAs, the units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:50 to 1:100,000 and the titer ascertained based on multiple point analysis.

Results

In Vivo Anti-gp39 Treatment Prevents the Formation of Germinal Centers in Immune Animals. It has been established that germinal center formation is a T cell–dependent process (23–26). Moreover, in vitro studies have suggested that the CD40 molecule may also play a role in the germinal center reaction (27, 32). Therefore, experiments were designed to determine whether the ligand for CD40, gp39, was required for the antigen-induced formation of germinal centers. Using an Ab specific for gp39, we examined the effect of blocking gp39–CD40 interactions on germinal center formation. Animals were immunized with SRBC, a TD antigen, and treated with anti-gp39 or control HIg on days 0, 2, and 4. On days 9 and 11, immunohistochemical staining was performed on spleen sections from both nonimmune and immune, treated animals. Germinal center formation was assessed by expression of PNA and mδ.

B cells undergo characteristic antigen-induced transitions within the follicle as a result of antigen exposure (for a review see reference 33). After immunization, antigen-specific

B cells undergo extensive proliferation followed by transition from a $m\delta^+$ phenotype to $m\delta^-$ (21, 34). Furthermore, germinal center B cells attain the ability to bind high levels of the lectin PNA, in contrast to follicular B cells which remain PNA^- (14, 21, 35). Fig. 1 (*left*) depicts splenic sections from SRBC-immune mice treated with control HIg and stained with PNA (*A*) or anti-δ (*C*). Immune mice develop extensive germinal centers within the primary follicles. These germinal centers are composed of large clusters of actively proliferating B cells expressing high levels of PNA binding sites (*A*). Within the germinal center, a peripheral region of $m\delta^+$ B cells surrounds the actively proliferating PNA^+ B cells (*C*). Treatment with anti-gp39, at the time of immunization, results in the complete inhibition of germinal center formation (*right*). Splenic sections from anti-gp39–treated animals showed almost complete absence of PNA-binding cells (*B*). Although some PNA-binding was visible in the stromal elements of the splenic mantle zone area, this staining pattern was also observed in animals treated with control Ab. Analysis of the $m\delta^+$ staining also confirmed the lack of germinal centers in anti-gp39–treated animals. As seen in Fig. 1 *D*, large primary follicles composed of $m\delta^+$, resting B cells are present in the spleens of animals treated with anti-gp39. This is in contrast to the PNA^+, $m\delta^-$ germinal centers within secondary follicles observed in HIg-treated immune animals.

A quantitative analysis of primary follicles and secondary follicles containing germinal centers present in immune mice treated with either control HIg or anti-gp39 is summarized in Table 1. Splenic sections from animals treated as described in Fig. 1 were analyzed quantitatively for $m\delta^-$, PNA^+ germinal centers as well as $m\delta^+$ primary follicles. The data are expressed as the number of germinal centers or primary follicles visible in seven splenic sections. Table 1 demonstrates

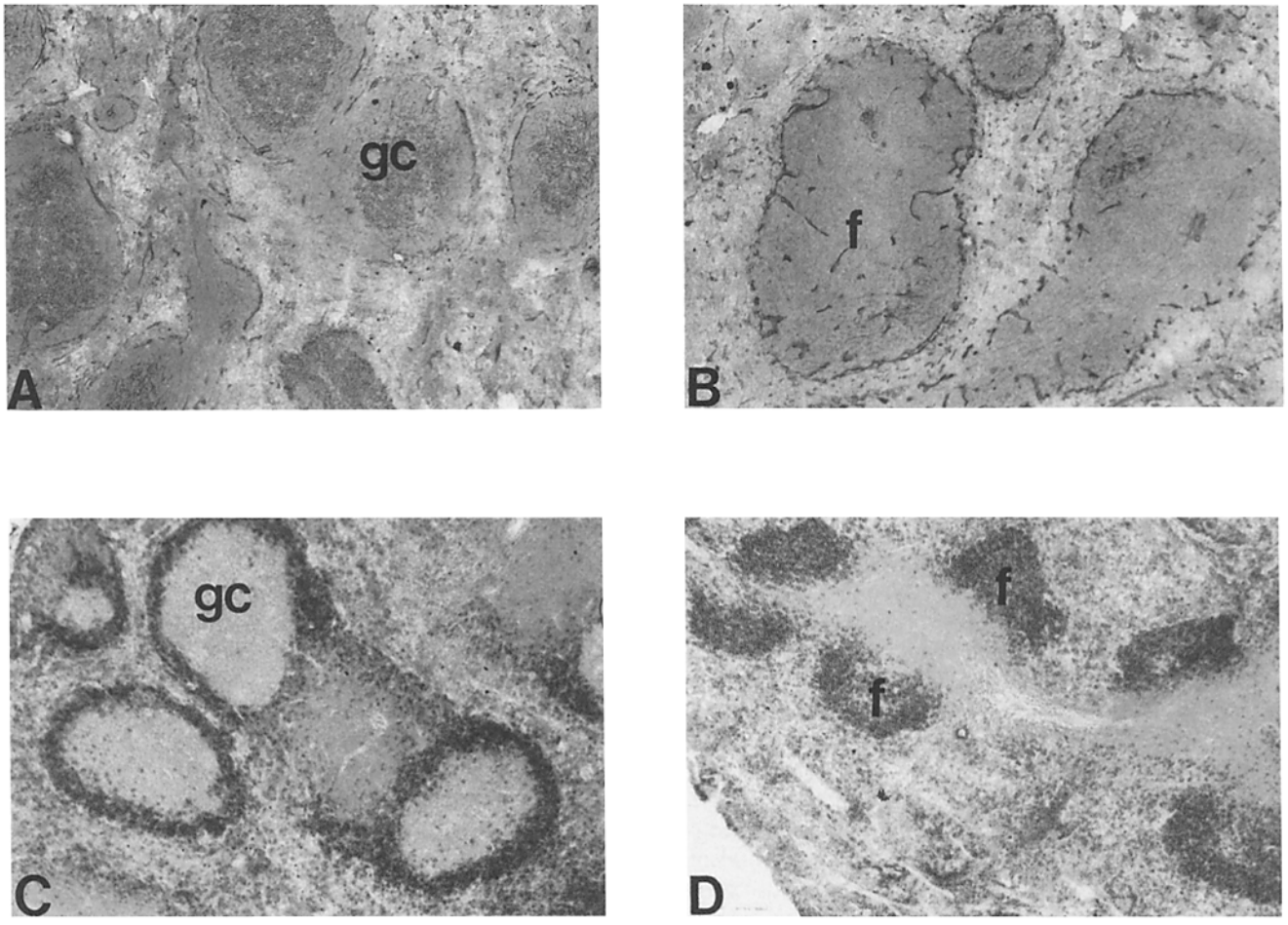

Figure 1. In vivo anti-gp39 treatment prevents the formation of germinal centers. Mice (three to four per group) were immunized with 100 μl of 10% SRBC i.p. on day 0. On days 0, 2, and 4 mice were treated with 250 μg of hamster Ig (*A* and *C*), or anti-gp39 (*B* and *D*). Spleens were removed on day 9 or 11 and immunohistochemistry was performed. Sections were stained for PNA (*A* and *B*) to detect PNA-binding germinal center memory B cells, or IgD (*C* and *D*) to visualize IgD^+ follicular B cells. In control-treated animals (*A* and *C*), large clusters of brightly PNA-staining B cells comprising the germinal centers are visible. IgD^+ follicular B cells are displaced into the peripheral zone around the actively proliferating germinal center B cells. In anti-gp39–treated animals (*B* and *D*), very few PNA-staining B cells are visible and no full-size germinal centers are present. Primary follicles containing almost entirely IgD^+ B cells are abundant with only rare follicles transforming to germinal centers. Primary follicles (*f*) and germinal centers (*gc*) are noted. (*A* and *C*) ×20, (*B*) ×25, and (*D*) ×10.

Table 1. *Anti-gp39 Treatment Prevents the Formation of Germinal Centers*

Treatment* (days after immunization)	PNA‡ (germinal centers)	IgD+§ (primary follicles)	IgD− (germinal centers)
Control (day 9)‖	63.5 ± 13.5¶	37.0 ± 6.1	65.5 ± 9.9
Control (day 11)	53.0 ± 22.9	46.7 ± 7.6	50.3 ± 30.0
Anti-gp39 (day 9)	3.0 ± 3.5	96.7 ± 17.6	6.0 ± 4.6
Anti-gp39 (day 11)	5.7 ± 8.1	97.7 ± 6.8	18.3 ± 9.5

* Mice (three to four per group) were immunized with 100 μl of 10% SRBC on day 0 and treated with control (HIg) or anti-gp39 (250 μg/injection) on days 0, 2, and 4.
‡ PNA-binding was used for the detection of germinal center B cells.
§ Anti-IgD was used for the detection of primary follicles (mδ+).
‖ Spleens were removed on day 9 or 11 and immunocytochemistry was performed on frozen sections.
¶ The data are representative of two experiments in which three to four animals were analyzed per group and are expressed as the mean number of germinal centers or follicles detected in seven frozen sections of 8-μm thickness.

that anti-gp39 treatment drastically decreased the number of PNA+, mδ− germinal centers that form as a result of immunization with sheep erythrocytes. Moreover, mδ staining indicates that the number of primary follicles present in anti-gp39–treated animals is significantly greater than that observed in control-treated animals, indicating an absence of follicular B cells transforming to germinal center, memory B cells. Overall, splenic sections from immune animals treated with anti-gp39 were indistinguishable from those of nonimmune animals (data not shown). Taken together, immunohistochemical analysis demonstrates that anti-gp39 treatment inhibits the formation of germinal centers within the spleens of immunized animals.

To establish that anti-gp39 treatment does not interfere with the capacity of follicular dendritic cells (FDC) to trap immune complexes, a process thought to be crucial for induction of B cell memory, mice were treated with anti-gp39 on days 0, 2, and 4 before administration of peroxidase–antiperoxidase immune complexes (200 μl i.v.) on day 5. Spleens were taken for immunohistochemistry on day 6, and trapped immune complexes were revealed by DAB-staining. No differences in the pattern of immune complex trapping in the spleen were found between animals treated with anti-gp39 or control HIg (results not shown). These results demonstrate that the immune complex trapping function of FDC is not impaired by anti-gp39.

In Vivo Anti-gp39 Treatment Inhibits the Generation of B Cell Memory in Immune Mice. Germinal centers are believed to be the site of generation of memory B cells within the splenic B cell compartment (19–21). Although the previous experiment showed that anti-gp39 treatment inhibits the formation of PNA+ germinal centers, it is important to assess whether the generation of functional B cell memory is also inhibited. The definition of functional B cell memory is the ability of B cells to elicit a secondary Ab response upon challenge with soluble antigen after adoptive transfer (36–41). To assess the effect of anti–gp39 treatment on the generation of functional B cell memory, CB17 (Igh^b) mice were immunized with TNP-BSA and treated with HIg or anti-gp39. After 4 wk, splenic B cells from these animals were adoptively transferred into KLH-primed BALB/c (Igh^a) recipients. Animals were subsequently challenged with TNP-KLH and the level of donor-specific $IgG1^b$ anti-TNP Abs was determined. The results, shown in Fig. 2, demonstrate that B cells from untreated or HIg-treated animals immunized with TNP-BSA produce high levels of donor-specific $IgG1^b$ anti-

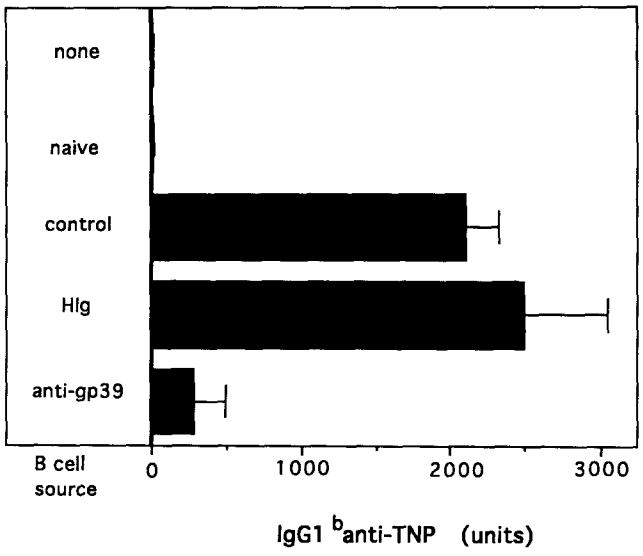

Figure 2. In vivo anti-gp39 treatment inhibits the generation of B cell memory. CB17 (Igh^b congenic strain of BALB/c) mice were immunized with TNP-BSA (100 μg in CFA) and administered anti-gp39 or HIg (on days 0, 2, and 4; 250 μg/d). After 4 wk, B cells were adoptively transferred into irradiated (600 rad) KLH-primed BALB/c recipients. Subsequently, animals were challenged with 10 μg soluble TNP-KLH, i.p. Serum $IgG1^b$ anti-TNP titers were ascertained on day 7 after transfer. Units represents arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titered from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The data are representative of two such experiments.

TNP Abs when transferred into KLH-primed recipient mice. However, the level of donor-specific IgG1^b anti-TNP Abs produced by animals which received B cells from animals treated with anti-gp39 was inhibited by >80%. This experiment confirms that the absence of germinal centers can be correlated to the lack of functional B cell memory, and demonstrates that blocking gp39–CD40 interactions directly inhibits the generation of functional memory B cells. These data, along with the immunohistochemical data, collectively support the tenet that gp39–CD40 interactions are critical in the formation of germinal centers as well as in the generation of B cell memory.

Discussion

The triggering of CD40 through its interaction with gp39 is essential for the generation of TD humoral immune responses in vivo (4, 5). This study examines the in vivo requirement for gp39–CD40 interactions in the formation of germinal centers and in the generation of B cell memory. In vivo administration of anti-gp39 prevented the formation of germinal centers as detected by immunohistochemical analysis of splenic sections from immune animals. Both the development of germinal center PNA-binding B cells and the transition of primary follicular mδ^+ B cells to mδ^- germinal center B cells were inhibited as a result of anti-gp39 treatment. Adoptive transfer of primed B cells from anti-gp39–treated mice demonstrated that blocking this interaction also resulted in a profound inhibition of Ab production by antigen-specific memory B cells.

Germinal centers arise in the follicles of secondary lymphoid tissue as a result of antigenic challenge. It is known that upon antigenic challenge, germinal centers become sites of extensive oligoclonal B cell proliferation (12, 13). Once activated, resting follicular mδ^+ B cells mature and proliferate to form large clusters of mδ^-, PNA-binding cells (14, 21, 34, 35). Somatic mutation, isotype switching, and antigen-specific B cell memory are all believed to occur within the specialized microenvironment of the germinal center (14–21). However, the precise sequence of signaling events required to drive the germinal center reaction are as yet unknown. The requirement for T cells in the formation of germinal centers has been demonstrated by examination of nude mice which fail to form germinal centers after antigenic challenge, but upon reconstitution with T cells, develop normal germinal centers (23). Although the requirement for T cells in the germinal center reaction seems clear, whether these T cells provide contact-dependent or lymphokine-mediated signals is as yet unresolved.

Several crucial pieces of evidence tend to support a role for gp39–CD40 interactions in the germinal center reaction. First, it is clear that signaling through CD40 either via anti-CD40 mAbs or its ligand results in extensive B cell proliferation, a primary event in the germinal center reaction. Second, several investigators have demonstrated in vitro that anti-CD40 Abs prevent B cells from undergoing programmed cell death within the germinal center (27, 32), demonstrating a role for CD40 in the survival of germinal center B cells. It has been suggested that this is a possible mechanism for rescuing germinal center B cells for the purpose of establishing a memory B cell pool (27). Finally, additional evidence that signaling through CD40 and its ligand, gp39, is critical for germinal center formation is provided by HIM patients. HIM patients lack the expression of gp39 or express mutant gp39 molecules which are incapable of CD40 binding (7–11). It is important to note that these patients lack germinal centers and functional B cell memory, suggesting that signals provided by gp39–CD40 interactions may be essential for these processes (6).

The hypothesis that gp39–CD40 interactions are critical for the development of B cell memory hinges on the fact that anti-gp39 does not delete or functionally alter Th cells. If either of these occur, then it is possible that the gp39–CD40 pair may only be indirectly involved in the generation of B cell memory. A number of lines of evidence strongly suggest that anti-gp39 does not alter Th cell number or function. First, antigen-specific Th cells from anti-gp39–treated animals can readily transfer helper function to recipients, indicating that Th cells are neither functionally silenced nor physically deleted as a consequence of in vivo anti-gp39 administration (4). Second, in situ, the frequency of IL-2–, IL-4–, and IFN-γ–producing T cells in immune anti-gp39–treated or untreated mice is identical, indicating that anti-gp39 does not diminish the frequency of lymphokine-producing T cells (5). Third, anti-gp39 does not diminish the humoral immune response to T-independent type II antigens, a response that is dependent upon endogenous lymphokine production for maximal responsiveness (4, 5). Fourth, anti-gp39 in vitro does not alter the proliferation of TCR transgenic T cells in response to the corresponding antigen, suggesting that anti-gp39 binding to gp39 does not exert a negative signal (Noelle, R. J., unpublished observation). Therefore, the mechanism of gp39 inhibition of memory is likely through the blockade of ligand binding to CD40. One additional assumption in this hypothesis is that gp39 expressed on activated T cells is responsible for the induction of B cell memory via binding to CD40 expressed on antigen-specific B cells. With the expression of gp39 on non-T cells (mast cells and basophils [42]) and CD40 on non-B cells (macrophages [43] and thymic epithelium [44]) other hypotheses are possible, but less likely.

The evidence provided in this study supports the hypothesis that gp39–CD40 interactions are critical for the generation of germinal centers and B cell memory. In vivo administration of anti-gp39 during antigen exposure results in profound inhibition of the formation of germinal centers within the spleens of immunized animals, as well as in the generation of functional memory B cells. The fact that in vivo anti-gp39 treatment probably inhibits Ab production in both primary and secondary responses through the blockade of gp39 binding to CD40, along with evidence indicating that antigen-specific and lymphokine-producing Th cells are not deleted after in vivo administration of anti-gp39, support the tenet that the processes of germinal center formation and memory B cell development are also prevented by blocking gp39–CD40–mediated signals.

The inhibition of gp39–CD40–dependent humoral immune responses has also been examined through the use of the soluble fusion protein CD40-Ig. Our laboratory has previously determined that in vivo administration of human CD40-Ig does not block the generation of primary Ab responses (Noelle, R. J., unpublished observation). This observation could be due to the low affinity of human CD40 Ig for murine gp39. However, a recent study (45) examining the effects of CD40-Ig in vivo demonstrated that soluble murine CD40-Ig did not block the generation of humoral immune responses or the formation of germinal centers. Lane et al. (45) showed that transgenic mice expressing serum levels of 10–30 μg/ml of murine CD40-Ig display normal Ig production and develop normal germinal centers in response to a soluble TD-antigen, an observation the authors attribute to the poor binding of the soluble monomeric CD40-Ig construct to the CD40 ligand, gp39. Consistent with the finding that CD40-Ig does not inhibit all gp39-dependent Ab responses, Gray et al. (46) demonstrate that in vivo administration of CD40-Ig does not block the formation of germinal centers. The fact that these authors do demonstrate inhibition of other gp39-dependent Ab responses suggests that the gp39–CD40 interactions involved in germinal center formation may be more stringent and therefore more difficult to block in vivo. Thus, it is likely that the failure of CD40-Ig to inhibit germinal center formation is due to the inefficient blocking ability of this reagent rather than the lack of a role for gp39 in germinal center formation. We believe that our data using a mAb previously shown to be efficient at blocking humoral responses in vivo (4, 5) provide strong evidence that gp39–CD40 interactions are essential for the development of germinal centers.

The histologic location at which gp39-expressing Th cells interact with resting B cells to initiate activation and differentiation to germinal center B cells is still unclear. Expression of gp39 on Th cells in situ after antigen administration has been shown to be restricted to the outer PALS and around the terminal arterioles (5). Although some gp39$^+$ Th cells have been detected in the follicles of human tonsils, lymph nodes, and spleen (47), gp39-expressing Th cells have not been detected in murine splenic germinal centers or marginal zones (5). These data indicate that the site of T-B interaction is likely to be in the PALS, suggesting that the initial site of B cell activation is outside the follicles. If this is the case, one could suggest that as a result of gp39–CD40 interactions, activated B cells migrate to the follicles, become competent to proliferate within the follicular microenvironment, and subsequently differentiate to form germinal centers.

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