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The Agonists of TLR4 and 9 Are Sufficient to Activate Memory B Cells to Differentiate into Plasma Cells In Vitro but Not In Vivo

Katharina Richard,* Susan K. Pierce,† and Wenxia Song2*  
Memory B cells can persist for a lifetime and be reactivated to yield high affinity, isotype switched plasma cells. The generation of memory B cells by Ag immunization requires adjuvants that generally contain TLR agonists. However, requirements for memory B cell activation and the role of TLRs in this activation are not well understood. In this study, we analyzed the response of memory B cells from immunized mice to TLR9 and 4 agonists CpG oligodeoxynucleotides (ODN) and LPS. Mouse memory B cells express both TLR9 and 4, and respond to both CpG ODN and LPS in vitro by differentiating into high affinity IgG secreting plasma cells. In contrast, neither CpG ODN nor LPS alone is sufficient to activate memory B cells in vivo. Ag is required for the clonal expansion of Ag-specific memory B cells, the differentiation of memory B cells to high affinity IgG secreting plasma cells, and the recall of high affinity Ab responses. The Ag-specific B cells that have not yet undergone isotype switching showed a relatively higher expression of TLR4 than memory B cells, which was reflected in a heightened response to LPS, but in both cases yielded mostly low affinity IgM secreting plasma cells. Thus, although memory B cells are sensitive to TLR agonists in vitro, TLR agonists alone appear to have little effect on B cell memory in vivo. The Journal of Immunology, 2008, 181: 1746–1752.

Immunological memory is a hallmark of adaptive immunity. The humoral branch of immunological memory consists of memory B cells, which are the precursors of high affinity Ab-secreting cells (ASCs),3 and long-lived plasma cells, which maintain serum Ab levels independent of antigenic stimuli. Recent studies have begun to characterize these two important humoral memory components (see Refs. 1–4). In the mouse system, to date, no distinct surface markers identify memory B cells. In general, memory B cells exhibit the characteristics of post-germinal center B cells. They express BCRs that have undergone isotype switching, somatic hypermutation, and affinity maturation, and thus bind to Ags with high affinities. IgM-expressing memory B cells with somatic hypermutation have been reported in human (5, 6); however, whether these cells are IgM-expressing memory B cells or marginal zone B cells remains controversial (7). Both long-lived plasma cells and memory B cells persist up to the lifetime of an individual as demonstrated by persistent Ab levels and the ability to mount rapid and robust Ab response to Ag challenge years after immunization. Memory B cells undergo rapid clonal expansion and differentiation to mount high affinity Ab responses upon exposure to Ags. Long-lived plasma cells are terminally differentiated and continue secreting Abs without antigenic stimulation in the bone marrow that provides the necessary environment for their longevity (4, 8, 9).

The requirements for the generation and maintenance of memory B cells has been studied in some detail (10). The generation of both memory B cells and long-lived plasma cells requires germinal center reaction, although germinal center-independent generation of memory B cells has also been reported (11, 12). Both Ag and the interaction of B cells with Th cells in the context of antigenic peptide-MHC class II complex and through CD40-CD40L are essential for the generation of germinal centers and memory B cells (13, 14). In contrast, the maintenance of the B cell memory seems to be independent of Ag and Th cells (15–17). Crotty et al. (15) reported that smallpox vaccine-specific memory B cells in human were detected 60 years postimmunization and 30 years after smallpox was eradicated worldwide. Maruyama et al. (17) showed that Ag-specific memory B cells persisted in transgenic mice where memory B cells switched their Ab specificity away from the immunizing Ag. Furthermore, mouse memory B cells have been shown to persist in the absence of Th cells (16) or follicular dendritic cells (18).

Although the requirements for the generation of B cell memory are becoming better understood, the requirements for the activation of memory B cells are still not clear. The rapid and robust responses of memory B cells to antigenic challenge suggest a low signaling threshold for memory B cell activation. Bernasconi et al. (19) reported that TLR agonists polyclonally activated human memory B cells to proliferate and differentiate into plasma cells in vitro, and that tetanus toxoid immunization increased serum Abs to unrelated Ags, suggesting that TLRs play a role in the polyclonal activation and long-term maintenance of human memory B cells in the absence of Ag in vivo.

The ability of common microbial products to increase the efficacy of immunization has long been observed (20). The discovery of TLRs reveals that some of the major components of commonly used adjuvant contribute to immune responses through TLRs.

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3 Abbreviations used in this paper: ASC, Ab-secreting cell; NP-KLH, 4-hydroxy-3-nitrophenyl acetyl-keyhole limpet hemocyanin.
TLRs are responsible for the initiation of innate immune responses and the maturation of dendritic cells that activate T cells, triggering adaptive responses (21, 22). The importance of TLRs in humoral immune responses was demonstrated using MyD88-knockout mice, where Th1 cell activation and T-dependent Ab responses were reduced or completely abolished (23). In addition to their role in activating Th cells, TLR agonists can directly act on B cells. Mouse naive B cells express TLRs, including TLR2, 4, 7, and 9, and proliferate and differentiate into plasma cells in response to TLR agonists (24–27). In contrast, human naive B cells do not express TLRs, but are induced to express TLRs in response to Ag stimulation through the BCR (19, 28). Human memory B cells constitutively express TLRs, including TLR2, 6, 7, 9, and 10, and proliferate and differentiate into plasma cells in response to TLR agonists alone in vitro. Using MyD88-knockout mice and LPS as adjuvant, Pasare and Medzhitov (29) demonstrated that TLR signaling in B cells was required for optimal Ab responses to T-dependent Ags. In contradiction, using MyD88−/−/Trif−/− mice, Gavin et al. (30) showed that both T cell-dependent and -independent Ags induced comparable humoral immune responses in the absence of TLR signaling. Thus, the exact role of TLR in the activation of humoral memory responses requires further examination.

In this study, we examined the role of TLR9 and 4 in the activation of memory B cells in vitro and in vivo using 4-hydroxy-3-nitrophenylacetyl-keyhole limpet hemocyanin (NP-KLH)-immunized mice as a model. We show that TLR4 and 9 agonists alone promote the differentiation of memory B cells into high affinity IgG ASCs in vitro; however, TLR agonists alone are not sufficient to effectively activate humoral memory responses in vivo.

Materials and Methods

Immunization

To generate humoral memory responses, C57BL/6 mice of 6–8 wk old (Charles River Laboratories) were immunized twice, 28 days apart, with 400 μg/mouse NP19-KLH (Biosearch Technologies) and Ribi adjuvant (BD Biosciences) to block FcγRs, followed by FITC-anti-mouse IgD, FITC-anti-mouse IgM, PE-anti-mouse CD138, PerCP-Cy5.5-anti-mouse B220 Abs and NP19-allophycocyanin in 2% PBS/PBS. After washing, cells were sorted using FACSaria (BD Biosciences) into four different populations: B220−/IgD−/IgM−/CD138− as plasma cells, B220+IgD+IgM−/CD138− as memory cells, B220+IgD−/IgM−/CD138− as NP-binding uncommitted B cells, and B220+IgD+IgM−/CD138− as non-NP-binding uncommitted B cells.

B cell in vitro stimulation

Splenic B cells isolated from mice 5 days or 6 wk after the second immunization and sorted plasma and B cell subpopulations were incubated at 5 × 10^6 cells/ml for 5 days with graded concentrations of CpG ODN, LPS, or Gardiquimod (InvivoGen) at 37°C, 5% CO2 before ELISPOT analyses.

ELISPOT analysis

Assay plates (Millipore) were coated with 10 μg/ml NP19-BSA, NP30-BSA (Biosearch Technologies), or anti-mouse IgG+M Ab (Jackson ImmunoResearch Laboratories) overnight at 4°C and blocked with 10% FBS in PBS for 2 h at 37°C. Cells were serially diluted into wells and incubated at 37°C for 5 h. Plates were washed with PBS and containing 0.05% Tween 20. Ab secreted by cells were detected with biotin-labeled anti-mouse IgM Ab (Jackson ImmunoResearch Laboratories) or a mixture of biotin-labeled anti-mouse IgG1, IgG2a, IgG2b, and IgG3 Abs (SouthernBiotech), followed by HRP-conjugated streptavidin (Kirkegaard & Perry Laboratories), and visualized by HRP substrate True Blue (Kirkegaard & Perry Laboratories). Plates were scanned by Cellular Technologies and spots were counted with software provided by Cellular Technologies.

ELISA analysis

ELISA plates (Nalge Nunc International) were coated with 40 ng/ml NP19-BSA, NP30-BSA (Biosearch Technologies), or 5 μg/ml anti-mouse IgG+M Ab (Jackson ImmunoResearch Laboratories) in 50 mM NaHCO3 (pH 9.6) overnight at 4°C and blocked with PBS containing 0.3% milk, 1% PBS, and 0.1% Tween 20 for 1 h at 37°C. Mouse serum was serially diluted into wells and incubated at room temperature for 2 h. After washing, the plates were incubated with a mixture of an equal amount of HRP-conjugated anti-mouse IgG1, IgG2a, IgG2b, and IgG3 Abs (SouthernBiotech), and visualized by 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma-Aldrich) and 0.03% hydrogen peroxide in phosphate-citrate buffer (pH 5.0). The reaction was stopped after 5 min with 0.5% SDS. Absorbance at 405 nm was measured using a plate reader (Wallac Victor, PerkinElmer). Titers were determined as highest serum dilution that gave a value that was ≥3 SD above the average reading for secondary controls.

Results

B cell responses to TLR4, 7, and 9 agonists in vitro

The ability of splenic B cells from NP-KLH-immunized mice to differentiate into NP-specific IgG and IgM high affinity ASCs when cultured with TLR4, 7, and 9 agonists was determined. Mice were immunized with 400 μg/mouse NP19-KLH plus Ribi adjuvant, a LPS-based adjuvant, twice, 4 wk apart. B cells were purified from spleens of NP-KLH-immunized mice 5 days after the second immunization and cultured with graded concentrations of CpG ODN or LPS for 5 days (Fig. 1, A–D). The number of NP-specific Ab ASCs was determined by ELISPOT using NP5-BSA as the coating Ag to capture only high affinity Abs and NP30-BSA to capture both high and low affinity NP-specific Abs. In the absence of CpG ODN or LPS in the culture medium, the splenic B cells did not differentiate into a significant number of NP-specific ASCs (Fig. 1, A–D). When cultured with CpG ODN or LPS, splenic B cells differentiated into NP-specific, IgG ASCs, a significant portion of which exhibited high affinity for NP (Fig. 1, A and B). CpG ODN induced relatively fewer NP-specific IgM ASCs (Fig. 1C).
described so far (1, 3), we defined Ag-specific memory B cells as IgG secreting cells in response to TLR4 and 9 agonists in vitro. B cells with memory phenotype differentiate into high affinity IgM ASCs.

Our results further showed that TLR4, 7, and 9 agonists induced ASCs from 5 days and 6 wk post immunization, but TLR4 agonist pre-exposed splenic B cells to differentiate into low affinity IgM ASCs. These results are consistent with previous reports that showed that TLR agonists can induce ASCs from high affinity IgM ASCs than LPS (Fig. 1D). However, the majority of these ASCs showed low affinity for NP (Fig. 1, C and D). The numbers of NP-specific ASCs generated in the B cell cultures peaked at 0.25 μg/ml CpG ODN and 0.6 μg/ml LPS (Fig. 1, A–D), suggesting that the stimulatory effect of TLR agonists CpG ODN and LPS was saturable. To test the presence of the precursor B cells for high affinity IgG ASCs in mice long after the immunization, splenic B cells from mice 6 wk after the second immunization were cultured with graded concentrations of CpG ODN, LPS, or Gardiquimod for 5 days. Similar to the splenic B cells isolated at 5 days (Fig. 1, A–D), when cultured with TLR4, 7, or 9 agonist, splenic B cells isolated at 6 wk post the second immunization were differentiated into NP-specific IgG ASCs, most of which exhibited high affinity (Fig. 1, E–G). TLR4 agonist induced a greater number of NP-specific IgM ASCs than TLR7 and 9 agonists; however, a significant portion of those exhibited low affinity (Fig. 1, H–J). These results are consistent with previous reports that showed that TLR agonists can stimulate the differentiation of B cells into plasma cells (25, 27). Our results further showed that TLR4, 7, and 9 agonists induced similar numbers of high affinity IgG ASCs from splenic B cells from 5 days and 6 wk post immunization, but TLR4 agonist predominantly activated splenic B cells to differentiate into low affinity IgM ASCs.

**B cells with memory phenotype differentiate into high affinity IgG secreting cells in response to TLR4 and 9 agonists in vitro**

Based on the general properties of memory B cells that have been described so far (1, 3), we defined Ag-specific memory B cells as isotype-switched, Ag-binding B cells (B220⁺IgD⁻IgM⁺CD138⁻NP⁺). Using flow cytometry, NP-specific memory B cells in the spleen were undetectable in nonimmune mice (frequency of 0.03%) (Fig. 2). Five days following the second immunization, the frequency of NP-specific memory B cells in the spleen reached ~0.5% of splenic B cells (Fig. 2). Six weeks after the second immunization, the frequency of NP-specific memory B cells in the spleen declined to ~0.1%, which was still higher than that of nonimmune mice. The frequency of NP-specific memory B cells remained at this level for several months (data not shown). The frequency of NP-binding, unswitched B cells (B220⁺IgD⁻IgM⁺CD138⁻NP⁺) was 1.1% in nonimmune mice, increased to 6.2% following immunization, and decreased to 1.6%, near nonimmune levels, by 6 wk following the second immunization (Fig. 2) in contrast to memory B cells. The relatively high frequency of these B cells in nonimmune mice as compared with the predicted frequency suggests that they may be very low affinity cells. These data show that immunization induced rapid increases in the frequencies of NP-specific B cells with the memory phenotype in the spleen. While the frequencies of memory B cells decreased with the time after the second immunization, a significant number of memory B cells persisted in the spleen for several months.

**FIGURE 2.** The frequencies of NP-specific memory B cells in the spleen of immunized mice. Splenic B cells were isolated from unimmunized mice (nonimmune) and mice 5 days and 6 wk post the second immunization with NP-KLH and Ribi adjuvant. Cells were stained with FITC-anti-IgD, FITC-anti-IgM, PE-anti-CD138, PerCP-Cy5.5-anti-B220 Abs and allophycocyanin-NP₁₉ and analyzed using a flow cytometer. Shown are representative data from eight independent experiments.
were not specific for NP. Population III, NP-binding unswitched B cells, might consist of Ag-experienced, unswitched B cells and marginal zone B cells specific for unrelated Ags. Before the in vitro culture, only population I, plasma cells, contained NP-specific ASC (Fig. 3, B and E); however, these did not survive in the culture (Fig. 3, C, D, F, and G). After culture in vitro with CpG ODN and LPS, B cells in population II, memory B cells, differentiated exclusively into NP-specific IgG ASCs, a large portion of which were high affinity (Fig. 3, C, D, F, and G). As expected, the NP-binding population of unswitched B cells generated a higher number of NP-specific IgM ASCs as compared with the non-NP-binding population (Fig. 3, F and G). Response of the unswitched B populations to LPS was nearly 10-fold greater than to CpG ODN (Fig. 3, F and G). These results demonstrate that TLR agonists can directly activate memory B cells to differentiate into high affinity, IgM ASCs.

**Differential expression of TLR4 and 9 in populations of B cells**

The differential responses of the B cell populations to TLR4 and 9 agonists shown in Fig. 3 suggest different expression levels of TLR9 and 4. To test this hypothesis, the levels of expression of TLR4 and 9 in the different populations of splenic B cells isolated from NP-KLH-immunized mice 5 days after the second immunization were determined by flow cytometry. The analyses showed higher TLR9 expression in NP-binding B cell populations (II and III) compared with naive B cells (population V) from nonimmune mice (Fig. 4, left panels). In contrast, the TLR4 surface levels of naive (population V) and unswitched B cells (populations III and IV) were significantly higher than that of NP-specific memory B cells (population II) (Fig. 4, right panels). These differences in expression levels provided an explanation for the equivalent response of memory B cells to CpG ODN and LPS and the hyper-response of NP-binding unswitched B cells to LPS. The activation of B cell memory responses by TLR4 and 9 agonists in vivo

To determine whether TLR4 and 9 agonists can stimulate memory B cells in vivo, mice were immunized with NP19-KLH plus Ribi adjuvant twice, 4 wk apart, to establish a pool of NP-specific memory B cells. Six weeks later, the mice were challenged with NP19-KLH, Ribi adjuvant, CpG ODN, LPS alone, or NP-KLH plus either Ribi adjuvant, CpG ODN, or LPS. Five days after the challenge, high and low affinity serum titers of NP-specific IgG and the number of high affinity, NP-specific IgG ASCs in spleens were determined. A certain level of high affinity serum titer (Fig. 5A) and a small number of high affinity, NP-specific IgG ASCs...
creased (10-fold) the serum titer of high affinity NP-specific IgG after immunization. The challenge with Ag alone significantly improved persistence of NP-specific IgG ASCs in the spleen even 6 wk after immunization (Fig. 5B), indicating the persistence of memory B cells in spleen long after an immunization. The number of high affinity IgG ASCs, with serum titers of high affinity NP-specific IgG or the number of high affinity NP-specific IgG ASCs in comparison with those before the challenge (Fig. 5, A and B). In contrast, challenging with Ag plus adjuvant dramatically increased the number of high affinity IgG ASCs, with ~56-, ~46-, and ~16-fold increases for Ag plus Ribi, LPS, and CpG ODN, respectively (Fig. 5B). LPS or Ribi appeared more effective than CpG ODN as the adjuvant under this specific immunization condition. At 5 days post the third immunization, the serum titers of mice challenged with CpG or LPS plus Ag were significantly higher than those that did not received the third immunization (None). The percentage of NP-specific memory B cells (Fig. 5A and B). This did not completely mirror what was observed for the numbers of NP-specific ASCs (Fig. 5B) and may reflect different kinetics and timing for B cell differentiation and for NP-specific plasma cells to exit from spleens in mice challenged with Ag alone and Ag plus adjuvant.

Taking advantage of the ability of memory B cells to specifically differentiate into high affinity IgG ASCs in culture with CpG ODN, we estimated the relative frequencies of NP-specific memory B cells in spleen after the different challenges. A significant number of NP-specific, high affinity IgG ASCs was detected in the 5-day culture of B cells from mice rested for 6 wk without the challenge (Fig. 5C), indicating the persistence of memory B cells in the spleen long after an immunization. The number of high affinity IgG ASCs differentiating from B cells from mice challenged with Ag or adjuvant alone was similar to or lower than that of B cells from mice that were not challenged (Fig. 5C). The percentage of NP-specific memory B cells among the splenic B cells was determined by flow cytometry, as described in Fig. 2. Shown are the representative results of four independent experiments.
Discussion

Humoral memory responses consist of two major components, long-lived plasma cells that secrete high affinity Ab and memory B cells, the precursors of long-lived plasma cells. In this study, we examined the role of TLR agonists in activation of humoral memory responses in vitro and in vivo by tracking these two major components of the humoral memory responses. We found that TLR4, 7, and 9 agonists could directly stimulate the differentiation of memory B cells into high affinity plasma cells in vitro; however, TLR agonist alone was not sufficient to stimulate the clonal expansion and differentiation of memory B cells and specific Ab recall responses in vivo.

Our study showed that when cultured with TLR4, 7, and 9 agonists, the splenic B cells from NP-KLH-immunized mice, either 5 days or 6 wk post the second immunization, differentiated into NP-specific ASCs with most of IgG ASCs as high affinity and most of IgM ASCs as low affinity. Using sorted memory B cells, we further showed that among different populations of B cells cultured with TLR4 or 9 agonist, NP-specific memory B cells exclusively differentiated into high affinity, isotype switched ASCs, and NP-binding unswitched B cells mainly differentiated into low affinity IgM ASCs. This demonstrates that TLR agonists can directly act on memory B cells in vitro and polyclonally stimulate the differentiation of memory B cells into high affinity, isotype switched plasma cells in the absence of T cells. In contrast, TLR agonists stimulated unswitched B cells in vitro primarily into low affinity, unswitched ASCs. Specific differentiation of sorted memory B cells into high affinity IgG ASCs indicates that they are the precursors of high affinity plasma cells and confirms that B cells sorted based on the phenotypic characteristics consist of mainly Ag-specific memory B cells. Furthermore, the differentiation specificity in the in vitro culture with TLR agonists provides us a good tool to determine the frequency of memory B cells in a mixture of multi-subpopulations of B cells. The finding that splenic B cells isolated from mice 5 days and 6 wk post the immunization responded to TLR4 and 9 agonists in a similar manner suggests the persistence of Ag-specific memory B cells in mice.

The experimental definition of memory B cells is still controversial, especially in the murine system. McHeyzer-Williams et al. (31, 32) established a multicolor flow cytometry method to follow NP-specific memory B cells in NP-KLH-immunized mice based on their phenotypic characteristics of post-germinal center B cells (B220<sup>+</sup>IgD<sup>-</sup>IgG1<sup>-</sup>NP<sup>-</sup>) and demonstrated that B cells with such phenotypes exhibited a high frequency of point mutations in their variable regions. Anderson et al. (33) recently suggested that CD80 and CD35 expression levels could be used to define B cell subtypes that have or have not undergone somatic hypermutation. Slifka and Ahmed (34) established an in vitro culture system to determine the frequency of acute lymphocytic choriomeningitis virus-specific memory B cells using the combination of limiting dilution and ELISPOT. Taking advantage of the ability of CpG ODN to stimulate the differentiation of memory B cells into high affinity IgG ASCs in vitro, we determined the frequency of memory B cells using this in vitro culture system in addition to flow cytometry. Both flow cytometry analysis and the in vitro culture system showed dramatic increases in the number of Ag-specific memory B cells 5 days after the second immunization and maintenance of a small, but significant, number of memory B cells 6 wk after the second immunization.

In addition to NP-specific, isotype switched B cells, a population of NP-binding, unswitched B cells were found in the immunized mice. This population of B cells was expanded in response to the second immunization, but returned to the basal level of unimmunized mice by 6 wk. When cultured with TLR4 and 9 agonists, they differentiated into NP-specific IgM ASCs, most of which exhibited low affinity for the Ag. Although the exact nature of the NP-specific unswitched B cells is unclear, it may contain IgM-expressing memory B cells. The existence of IgM-expressing memory B cells is still controversial. The discovery of a subpopulation of CD27<sup>+</sup>IgM<sup>+</sup>, somatic mutated B cells in human peripheral blood (5) suggests the presence of IgM expressing memory B cells. Weller et al. (7) showed that this population of B cells was circulating spleen marginal zone B cells that mutated their Ig receptors during ontogeny, before their differentiation into T-independent Ag-responsive cells. The role of this population of B cells in humoral memory responses remains to be further defined.

Our studies present a first comparison of B cell responses to TLR4 and 9 agonists in vitro and in vivo. Our results showed that TLR4 and 9 agonists were equally effective in stimulating memory B cells to differentiate, but TLR4 agonist was much more efficient than TLR9 agonist in stimulating unswitched B cells from immunized mice to differentiate in vitro. This differential response can be explained by different expression levels of TLR4 and 9 in different subpopulations of B cells. We found that the immunization increased the expression levels of both TLR4 and 9 in mouse B cells, and the expression level of TLR9 was maintained and the expression of TLR4 down-regulated when activated B cells differentiated into memory B cells. Our finding of an increase in TLR9 expression level by immunization is similar to the report of Bernasconi et al. (28) that human memory B cells, but not naive B cells, constitutively express TLR9. The biological significance of this differential expression of TLR9 and 4 among different B cell subpopulations remains to be explored. The higher expression level of TLR4 in naive and activated, unswitched B cells than memory B cells predicts that LPS preferentially induces primary humoral responses.

The direct stimulatory effect of TLR9 and 4 agonists on memory B cells in vitro showed in this study and previous studies (19, 28) suggests their potential role in polyclonal activation of memory B cells in vivo. Our finding that TLR9 or 4 agonist alone failed to increase the frequencies of either Ag-specific high affinity IgG ASCs or Ag-specific memory B cells, as well as the concentration of Ag-specific IgG in serum, does not support the polyclonal activation hypothesis. Our result that Ag in combination with TLR9 or 4 agonist was more effective than Ag alone for inducing clonal expansion and plasma cell differentiation suggests that the activation of memory B cells in vivo still requires more than one signal, Ag-triggered BCR-mediated signal in combination with TLR-triggered signals or T cell help. The findings that on day 5 post the third immunization, Ag alone induced a higher serum titer but a lower frequency of high affinity NP-specific IgG ACS in spleens than Ag plus CpG or LPS may reflect differences in the kinetics of B cell differentiation and plasma cell migrating from the spleen to the bone marrow in response to different stimuli. Some other factors may limit the direct role of TLRs on B cells, such as the competition between B cells and dendritic cells to interact with TLR agonists. TLR agonists are very effective in the induction of dendritic cell maturation. Dendritic cell-activated T<sub>H</sub> cells provide memory B cells with a second stimulation signal. Above all, requirement of both Ag and TLR agonists for the activation of memory B cells in vivo ensures the specificity of humoral memory responses.
The necessity of TLRs in humoral memory responses is controversial. Disruption TLR signaling by knocking out MyD88 and/or Trif genes either did or did not significantly inhibit Ab responses against T-dependent Ags, depending on the specific experimental system (23, 29, 30). The study presented here reveals a direct stimulatory role of TLR on memory B cell differentiation in vitro and the requirement of both Ag and TLR agonist for effective activation of humoral memory responses in vivo. These results strongly support a critical role for TLRs in recalling humoral memory responses.

Disclosures
The authors have no financial conflict of interest.

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