Novel Role of the Ras Cascade in Memory B Cell Response

Yoshimasa Takahashi,1 Ayako Inamine,1,2 Shu-ichi Hashimoto,1 Sachiko Haraguchi,1 Emi Yoshioka,1,4 Naoya Kojima,4 Ryo Abe,2,3 and Toshitada Takemori1,*

1Department of Immunology
National Institute of Infectious Diseases
1-23-1 Toyama
Shinjuku
Tokyo 162-8640
Japan
2Division of Immunobiology
Research Institute for Biology Science
Science University of Tokyo
2669 Yamazaki
Noda
Chiba 278-0022
Japan
3Genome and Drug Research Center
Science University of Tokyo
2669 Yamazaki
Noda
Chiba 278-0022
Japan
4Institute of Glycotechnology and Department of Applied Biochemistry
Tokai University
1117 Kitakaname
Hiratsuka
Kanagawa 259-1292
Japan

Summary

Engagement of the B cell antigen receptor (BCR) triggers the Ras cascade, but the biological role of the latter in B cell response is unknown. Here, we report that in T cell-dependent response, the role of the Ras cascade is confined to memory B cells and possibly marginal zone B cells. When Ras-dependent BCR signaling was impaired, the generation of IgG germinal center B cells was unaffected but the recruitment of high-affinity cells into the memory compartment and terminal differentiation were inhibited. Furthermore, inhibition of MEK activity consistently impaired antibody production by IgG memory B cells (but not naïve B cells) in vitro. Notably, this impairment was countered by overexpression of Bcl-2. Thus, our data suggest that upon antigen stimulation, memory B cells are susceptible to apoptosis but can be rescued via an antiapoptotic effect mediated through the Ras cascade.

Introduction

Memory B cells acquire several unique intrinsic properties during development, which distinguish them from naïve B cells. For example, longevity, preferential localization to the antigen-draining sites, and altered expression of cell-surface molecules including isotype-switched BCR (Schittek and Rajewsky, 1990; McHeyzer-Williams et al., 1991; Liu et al., 1995; Rajewsky, 1996; Arpin et al., 1997; Klein et al., 1998; Ridderstad and Tarlinton, 1998; Tangeye et al., 1998; Takahashi et al., 2001). Stimulation of IgG+ memory B cells with a minute amount of recall antigen causes prompt and robust antibody production (Arpin et al., 1997). The IgG BCR has a C-terminal cytoplasmic domain that is structurally distinct from those of IgM and IgD. If the cytoplasmic domain of the IgM BCR is replaced with an IgG tail, antibody production upon stimulation with T cell-dependent antigen is enhanced (Martin and Goodnow, 2002). This suggests that the biological activity mediated by the IgG BCR differs from that of the IgM BCR. However, it is unknown how the IgG BCR complex on memory B cells transmits signals differently than the IgM BCR complex on naïve B cells.

The BCR complex mediates multiple signal cascades through the Ras and signalosome pathways (Niño and Clark, 2002). The signalosome consists of Burton's tyrosine kinase (Btk), BLNK/SLP-65/BASH, Vav, and phospholipase C-γ2 (PLCγ2). Disruption of BLNK, PLCγ2, or PI3K results in B cell deficiency with decreased numbers of mature B cells and attenuated response to a type II T cell-independent antigen. (Fruman et al., 1999; Suzuki et al., 1999; Xu et al., 2000; Wang et al., 2000; Doody et al., 2001). The inherited X-linked deficiency with Btk mutations leads to a poor antibody response to a type II T cell-independent antigen, poor GC development, low memory B cell generation, and insufficient affinity maturation during the primary response to T cell-dependent antigen, although it does not affect the secondary response (Ridderstad et al., 1996).

Ras is a 21 kDa guanine-nucleotide protein with three isoforms (Kolch, 2000; Chang et al., 2003). It has been suggested that IgM BCR might use two guanine nucleotide-exchange factors (RasGEFs) to activate Ras: a RasGEF homologous to the Drosophila Son of sevenless (Sos) and a Ras guanine nucleotide-releasing protein (RasGRP) (Harmer and DeFranco, 1997; Oh-hora et al., 2003). Although pharmacological inhibition of the Ras-MEK pathway partially impairs B cell proliferation in response to anti-IgM stimulation in vitro (Richards et al., 2001), it is unknown whether the Ras-MEK cascade plays any role during the B cell response in vivo.

To investigate whether BCR signaling via the Ras cascade mediates a unique effect on the B cell response, we examined the response of transgenic (TG) mice to T cell-dependent antigen stimulation. The TG mice chosen for the study express a dominant-inhibitory mutant of Ha-ras (Asn-17 Ha-ras, [Feig and Cooper, 1988]), preferentially in B lineage cells (Nagaoka et al., 2000). Asn-17 Ha-ras has proved to be extremely valuable as a tool to probe Ras functions [Feig and Cooper, 1988; Iritani et al., 1997; Nagaoka et al., 1998; Matallanas et al., 2003], as the mutant efficiently diminishes the activity of all three isoforms of Ras (Matalla-
Inhibition of Ras Activity Does Not Affect the Primary B Cell Response to T Cell-Dependent Antigen

TG mouse lines were established previously on the C57BL/6 background and designated N-17-95 TG and N-17-52 TG. These mice express a high level of Asn-17 Ha-ras in B lineage cells but only minute levels in T lineage cells (Nagaoka et al., 2000). The expression of an inhibitory Ras mutant caused a significant reduction in ERK activation and splenic B cell proliferation after stimulation with anti-IgM Abs (see Figure S1 in the Supplemental Data available with this article online).

We determined the frequency of transitional, follicular (FO), and marginal zone (MZ) B cells in TG and wild-type (wt) mice by flow cytometry (Loder et al., 1999; Allman et al., 2001), observing that constitutive expression of Asn-17 Ha-ras reduced the number of immature transitional B cells (T1, T2, and T3 stages) 10-fold (Figure S2). This decrease probably reflects a developmental arrest at the transition from pre-pro-B cells to pre-B cells in the bone marrow (Nagaoka et al., 2000). The numbers of FO B cells in TG mice were reduced to ~28% of wt controls, whereas the effect on MZ B cells was less extensive (Figure S2). Accumulation of 5'-bromo-3'-deoxyuridine (BrdU) by MZ and FO B cells in both TG and wt mice was comparable at 24 hr and 7 days after pulse (Figure S3A). Moreover, BrdU-labeled cells in TG and wt mice persisted at similar levels for 15 days (Figure S3A), suggesting that expression of Asn-17 Ha-ras has little effect on the development of the splenic B cell compartment from dividing precursors and the life spans of MZ and FO B cells.

Immunization with (4-hydroxy-3-nitrophenyl)acetyl (NP) coupled to chicken γ-globulin (CG) in alum elicited comparable titers of NP-specific IgM and IgG Ab in N-17-95 TG, N-17-52 TG, and wt mice at day 10 after priming (Figure 1A). With time, high-affinity IgG Ab titers increased with similar kinetics in both TG and wt mice (Figure 1C). In addition, frequencies of Ab-forming cells (AFCs) in BM that secreted high-affinity, NP binding IgM, Ab were comparable among N-17-95 TG, N-17-52 TG, and wt mice 120 days after immunization (Figure 1D).

Given that MZ and FO B cells can mount primary IgG Ab responses to T cell-dependent antigen (Song and Cerny, 2003), we assessed the contribution of each B cell subset in the primary response of TG mice. Naïve B cells enriched for or depleted of FO B cells were mixed with CG-primed CD4 T cells from wt mice and transferred into C57BL/6-scid recipients that were subsequently immunized with NP-CG in alum. As shown in Figures 1E and 1F, B cells enriched for FO B cells from TG and wt mice mounted similar anti-NP IgM and IgG Ab responses, suggesting that Ras has little effect on primary Ab responses by FO B cells. As previously reported (Song and Cerny, 2003), efficient primary Ab responses were produced by wt splenic B cells depleted of FO B cells, but not by TG B cells depleted of FO B cells. This result suggests that the Ras cascade is involved in MZ B cell activation and/or differentiation driven by T cell-dependent antigen, even though the cascade has little effect on the survival of resting MZ B cells.

To examine the effects of Ras activity on the GC reaction and humoral memory, we followed the kinetics of NIP binding GC B cells (B220+CD38lowIgG1+,lowIgM+IgD−CD43−) and memory B cells (B220+CD38+IgG1+highIgM−IgD−CD43−) by using flow cytometry (Figure 2A) (Takahashi et al., 2001). In TG and wt mice, numbers of NIP binding IgG1+GC B cells in the spleen peaked at day 8 postimmunization (Figure 2C). Similarly, NIP binding memory B cells reached their peak at day 20 postimmunization and retained this number for long periods in TG and wt mice (Figure 2D). GC B cells from both TG and wt mice incorporated significant amounts of BrdU at day 40 postimmunization, whereas there was little or no BrdU incorporation by TG and wt memory B cells at this time (Figure S3B). These results suggest that the persistence of memory B cells in TG and wt mice is associated with increased life span rather than continuous renewal.

Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) revealed that bcl-2, bcl-xL, and Fas were expressed at comparable levels in the B cell subpopulations of both TG and wt mice (Figure S4). Owing to the expression of Asn-17 Ha-ras in TG mice, the expression level of ras was upregulated equally among their naïve, GC, and memory B cells. Analysis by flow cytometry showed that the expression of Fas (CD95), CD21, CD22, CD40, CD80, and CD86 on GC and memory B cells was similar in TG and wt mice (Figure S5). Together, these results strongly indicate that Ras is not required for the expansion of GC B cells or for the generation and maintenance of memory B cells.

Impaired Affinity Maturation in Ras-Defective Memory B Cells

At 20–40 days after immunization, TG and wt GC B cells accumulated equal frequencies of point mutations and among these were comparable numbers of replacement (tryptophan to leucine) mutations at position 33 in CDR1 (Leu33) (Figure 3A, Table 1). The Leu33 substitution generally results in a 10-fold increase in affinity for NP (Allen et al., 1988); the equivalent frequencies of this recurrent, affinity-enhancing mutation suggest that selection for high-affinity GC B cells is normal in TG mice. Mutation frequencies in memory B cells from TG and wt mice were lower than those of GC B cells (Figure 3B), and a minor population of memory B cells carried unmutated V_{H}186.2 rearrangements. Unmutated B cells with a memory phenotype suggest generation before the onset of somatic mutation in the GC or an origin independent of the GC reaction (McHeyzer-Williams et al., 1991; Takahashi et al., 2001; Toyama et al., 2002; Inamine et al., 2005). No significant difference in the number of somatic mutations in memory B cells was observed between TG and wt mice (Figure 3B). However, 40–120 days after immunization, the frequency of the Leu33 replacement mutations in TG memory B cells...
Figure 1. Effects of Impaired Ras Activity on the Primary Antibody Response to T Cell-Dependent Antigen

(A) Wt (white bars), N-17-95 TG (gray bars), and N-17-52 TG (striped bars) mice were immunized with NP-CG/alum and then anti-NP IgM, and IgG1 Ab titer was determined by ELISA at day 10 postimmunization. Each circle represents the result for an individual mouse.

(B and C) Total and high-affinity anti-NP IgG1 Ab titers in wt (open circles), N-17-95 TG (closed circles), and N-17-52 TG (closed squares) mice were determined by ELISA by using NP18-BSA (B) or NP3-BSA (C). Results are shown as mean ± SD from each group (n > 7).

(D) At day 120 postimmunization, BM cells were plated for ELISPOT assay with NP3-BSA or NP18-BSA as the capture antigen.

(E and F) FO B cells and non-FO B cells were enriched from wt (open circles) and N-17-95 TG (closed circles) mice and transferred into scid mice together with CG-primed CD4 T cells. Fourteen days after priming with NP-CG/alum, anti-NP IgM (E) and IgG1 (F) Ab titer was determined by ELISA with NP18-BSA. An asterisk indicates that the results are significantly different between TG and wt mice (p < 0.05). The data are representative of two independent experiments.

was 4- to 8-fold lower than in wt memory B cells. (Figure 3B, Table 1). By contrast, the frequency of high-affinity clones with the Leu33 substitution in NP-specific BM AFCs was comparable between TG and wt mice at day 120 postimmunization (Figure 3B, Table 1). We interpret these data to indicate that impaired Ras activity interferes with the recruitment of high-affinity precursors into the memory compartment, but not into the long-lived AFC compartment.

Ras-Defective Memory B Cells Do Not Respond to Secondary Immunization

To examine whether restimulated memory B cells may develop into plasma cells by way of B220+CD138+ intermediates (Shapiro-Shelef et al., 2003), we challenged N-17-95 TG and wt mice with soluble NP-CG. In wt mice, NP-specific IgG1+ AFC numbers were increased about 20-fold in the spleen 5 days after challenge (Figures 4A and 4B); however, CG-primed wt mice challenged with soluble NP-CG generated no or few AFCs at day 5 (data not shown), suggesting that the majority of AFCs detected are likely generated from memory B cells stimulated with recall antigen.

TG mice mounted anti-NP IgG recall responses, but Ab levels were 5- to 10-fold below that of wt mice. Further, the ratio of high-affinity AFC in secondary T response was reduced compared to wt controls (Figure 4C) along with the number of NIP binding IgG1+ B220−FO−MZ−T only splenocytes (Figure 4D). The majority of B220−FO−MZ−T only cells were CD138−CD43−CD11b+ (data not shown), a phenotype similar to the preplasma memory B cells of Shapiro-Shelef et al. (2003). In contrast to these differences, plasmacytes and preplasma memory B cell numbers were similar in the late primary response, before boosting (Figures 1D and 4A–4D). We conclude that during recall responses, Ras is required for the transition of memory B cells into preplasma memory and plasma cell compartments.

Memory B cell responses were examined further by adoptive-transfer experiments to minimize the influence of AFCs that were generated during the primary response. B cells were enriched from the pooled spleens of naïve or NP-primed TG and wt mice by depleting T cells, macrophages and granulocytes, and AFCs. The recovered B cells (>95% B220+) were mixed with T cells (>90% CD3+) from CG-primed wt mice and transferred into Rag-1−/− mice that were subsequently injected with soluble NP-CG. Recipients given NP-primed, wt B cells generated robust anti-NP responses with high-affinity IgG1, Ab (Figures 4E–4G). In contrast, mice reconstituted with naïve, wt B cells produced much lower (~500-fold) levels of NP-specific IgG1, Ab. Anti-NP humoral responses in mice given NP-primed, TG B cells were also significantly below that of animals reconstituted with primed wt cells. Levels of NP-specific IgG1, Ab were ~30-fold lower in recipients of NP-primed, TG B cells, and the average affinity of this Ab was approximately half of that produced by wt memory B cells (Figure 4G). Together, these data strongly support the idea that Ras activity is required for the recruitment of high-affinity cells into the memory compartment and for terminal differentiation of memory B cells.

Antigen Does Not Drive Ras-Defective Memory B Cells to Differentiate into AFCs In Vitro

To determine whether TG memory B cells are unresponsive because of deficient BCR signaling, memory B cells were purified from the spleens of TG and wt
Figure 2. Inhibition of Ras Activity Does Not Affect the Generation and Maintenance of GC B Cells and Memory B Cells

(A) Splenocytes were recovered from NP-CG-primed wt and N-17-95 TG (TG) mice at day 80 postimmunization and were stained. Tricolor and PI-negative cells were selected under a lymphocyte gate with forward and side light scatter. NIP binding/B220+/IgG1+ cells were gated and separated into memory (IgG1high/CD38+) and GC (IgG1dull/CD38dull) B cells, respectively. The number of gated cells per 1,000,000 events is listed on each panel.

(B) IgG1 versus NIP staining from naïve mice (Day 0), NP-CG-primed mice (Day 8 NP-CG and Day 95 NP-CG), or CG-primed mice (Day 8 CG) is shown.

C

D

Figure 2. Inhibition of Ras Activity Does Not Affect the Generation and Maintenance of GC B Cells and Memory B Cells

(A) Splenocytes were recovered from NP-CG-primed wt and N-17-95 TG (TG) mice at day 80 postimmunization and were stained. Tricolor and PI-negative cells were selected under a lymphocyte gate with forward and side light scatter. NIP binding/B220+/IgG1+ cells were gated and separated into memory (IgG1high/CD38+) and GC (IgG1dull/CD38dull) B cells, respectively. The number of gated cells per 1,000,000 events is listed on each panel.

(B) IgG1 versus NIP staining from naïve mice (Day 0), NP-CG-primed mice (Day 8 NP-CG and Day 95 NP-CG), or CG-primed mice (Day 8 CG) is shown.
Ras Function in Memory B Cell Response

Figure 3. Accumulation of Somatic Mutations in GC B Cells, Memory B Cells, and BM AFCs

(A) NIP binding GC B cells were sorted from the pooled spleens of wt (white bars) and N-17-95 TG (gray bars) mice at the indicated days after immunization with NP-CG.

(B) NIP binding memory B cells and \( \lambda \)-bearing AFCs (CD138+) were purified from the pooled spleens and BMs of the NP-primed mice: white bars, wt; gray bars, N-17-95 TG; striped bars, N-17-52 TG. Circles represent the number of mutations in individual \( V_{H} \) genes. Closed circles indicate the genes carrying a tryptophan-to-leucine substitution at position 33.

mice and stimulated in vitro with a type II T cell-independent antigen, NP-Ficoll (Maizels and Bothwell, 1985), or a mitogenic control, lipopolysaccharide (LPS) (McHeyzer-Williams et al., 1991). NIP binding IgG1+ memory B cells and NIP binding IgM+ naïve B cells were purified from the spleens of TG or wt mice and then cultured in medium containing IL-2, IL-4, and IL-5. The addition of LPS induced both memory and naïve B cells to form AFCs (Figures 5A and 5C), indicating that TG memory B cells are fully capable of terminal differentiation into AFCs. Supporting the results described in Figures 3 and 4 and Table 1, TG memory B cells produced lower-affinity IgG1 Ab in the culture fluid compared to wt memory B cells (NP3/NP18 ratio; 5.2 ± 4.4% versus 13.9 ± 2.0%, \( p = 0.0369 \)).

In vitro, NP-Ficoll triggered Ab production by wt memory B cells, whereas it induced little or no Ab production by TG memory B cells (Figure 5B). Interestingly, NIP binding IgM+ B cells from naïve TG mice responded to NP-Ficoll and produced significant amounts of IgM Ab, which were comparable to those secreted by wt NIP binding IgM+ B cells (Figure 5D). These results support the idea that BCR signaling through the Ras cascade is indispensable for antigen-driven terminal differentiation of IgG1+ memory B cells, but not of IgM+ naïve B cells. Together, these results indicate that the unresponsiveness of TG memory B cells to recall antigen might be due, at least in part, to impaired BCR signaling caused by Ras inhibition. To examine whether Ras is required for the transition of IgG1 memory B cells into AFCs, we analyzed the effects of the MEK inhibitor U0126 on Ab production by wt memory B cells in response to NP-Ficoll (Richards et al., 2001). MEK inhibitor reduced Ab production in a dose-dependent manner (Figure 5F) but had little effect on the IgG memory B cell response to LPS (Figure 5E) and the naïve IgM B cell response to NP-Ficoll (Figure 5H). These results suggest that BCR signaling mediated by the Ras-Raf-MEK cascade is required for the terminal differentiation of IgG memory B cells into Ig-producing plasma cells, but the cascade is not required for the response of naïve B cells.

Impaired Memory B Cell Response Is Countered by Overexpression of Bcl-2

The expression of transcriptional repressor B lymphocyte-induced maturation protein-1 (Blimp-1) is required for plasma cell development in the primary and memory B cell response (Calame, 2001; Shapiro-Shelef et al., 2003). Blimp-1 represses genes associated with B cell growth and proliferation while activating genes involved in Ig secretion (Calame, 2001; Shaffer et al., 2002). To investigate the linkage between Ras activity and Blimp-1 expression in memory B cell response, RT-PCR was used to analyze the level of Blimp-1 mRNA in memory B cells after stimulation with NP-Ficoll. Blimp-1 message levels comparably increased in both TG and wt memory B cells at 24 hr poststimulation (Figure 6A). The mRNA levels of the membrane and the secretory form of IgG1 were not changed during this time. Therefore, Ras is not associated with Blimp-1 expression during terminal differentiation.

Because the Ras cascade mediates an antiapoptotic effect (Kolch, 2000; Chang et al., 2003), it is possible that Ras inhibition might trigger memory B cell death upon BCR stimulation. To test this possibility, we investigated whether the defect in terminal differentiation could be countered by the antiapoptotic effect of Bcl-2 (Cory and Adams, 2002). After priming with NP-CG, splenic B cells were recovered from homozygous N-17-95 TG mice that coexpressed the bcl-2 transgene (N-17-95/Bcl-2 TG), from TG, and from wt and Bcl-2 transgenic controls. The recovered B cells (>95% B220+) were mixed with T cells (>90% CD3+) from CG-primed wt mice and transferred into Rag-1−/− mice, followed by challenge with soluble NP-CG. Because overexpression of BCI-2 in B lineage cells significantly increases the number of all B cell types, including memory cells (Smith et al., 1994 and data not shown), numbers of B cells transferred were adjusted to yield (C) NIP binding GC B cells and (D) NIP binding memory B cells were enumerated by flow cytometry from NP-CG-primed wt (open circles) and TG (closed circles) mice at the indicated times postimmunization. As a control, the numbers from CG-primed wt (open squares) and TG (closed squares) mice are plotted. Each symbol represents the number of cells in an individual mouse. The data are representative of two independent experiments.
Table 1. Ratios of \(V_h\) Genes Carrying Leu33 Mutations in Each B Cell Subpopulation

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Wt</th>
<th>TG N-17-95</th>
<th>TG N-17-52</th>
</tr>
</thead>
<tbody>
<tr>
<td>d20 GC</td>
<td>46.7</td>
<td>40.0 ((p = 1.0000)^b)</td>
<td>-</td>
</tr>
<tr>
<td>d40 GC</td>
<td>40.0</td>
<td>25.0 ((p = 0.6517))</td>
<td>-</td>
</tr>
<tr>
<td>d40 memory</td>
<td>50.0</td>
<td>7.7 ((p = 0.0516))</td>
<td>-</td>
</tr>
<tr>
<td>d88 memory</td>
<td>21.1</td>
<td>&lt;3.7 ((p = 0.0238))</td>
<td>-</td>
</tr>
<tr>
<td>d120 memory</td>
<td>42.9</td>
<td>10.5 ((p = 0.0341))</td>
<td>5.3 ((p = 0.0094))</td>
</tr>
<tr>
<td>d120 BM AFC</td>
<td>40.0</td>
<td>35.7 ((p = 1.0000))</td>
<td>35.7 ((p = 1.0000))</td>
</tr>
</tbody>
</table>

*a Data were based on the sequence analysis of the \(V_h\) gene as depicted in Figure 3.

*b The two-sided p value was determined by Fisher’s exact test between TG and wt mice.

an equal number of memory cells in each recipient group. As shown in Figure 6B, overexpression of Bcl-2 increased the anti-NP IgG1 response by Ras-deficient memory B cells to levels comparable with that of wt memory B cells but did not improve the affinity maturation (Figure 6C). The simplest explanation of the data is that Bcl-2 revises Ras activity during terminal differentiation, but not during recruitment of high-affinity cells into the memory compartment. Otherwise, considering that a high dose expression of Bcl-2 expands low-affinity memory B cells as a consequence of antiapoptotic effect on low-affinity GC B cells (Smith et al., 2000), we could not exclude the possibility that overexpression of Bcl-2 might rescue high-affinity memory cells in TG mice but that the rescued high-affinity cells might be masked by concomitant expansion of low-affinity cells.

To further confirm the effect of Bcl-2 on the secondary response by Ras-deficient memory B cells in vitro, purified memory B cells were stimulated in vitro with NP-Ficoll or LPS. Figure 6D shows that memory B cells from wt, N-17-95 TG, or N-17-95/Bcl-2 TG mice produced large amounts of IgG Ab in response to LPS. On the other hand, the coexpression of Bcl-2 in Ras-defective memory B cells rescued IgG antibody production in response to NP-Ficoll to a level comparable with normal memory cells at day 6 poststimulation (Figure 6E). Together, these results suggest that impaired IgG antibody production by Ras-defective memory B cells is due to apoptosis upon BCR stimulation.

**Discussion**

We demonstrate that expression of an inhibitory Ras mutant impairs the recruitment of high-affinity precursors into the IgG memory compartment and prevents terminal differentiation of IgG memory B cells in re-

![Figure 4. Inhibition of Ras Activity Impairs the Memory B Cell Response after Secondary Challenge](image-4.png)

NP-CG-primed wt and N-17-95 TG (TG) mice were boosted at day 102 postimmunization, and their splenocytes were recovered at day 5 postboosting. NP3-specific AFCs (A) and NP18-specific AFCs (B) were enumerated by ELISPOT from wt (white bars) and TG (gray bars) mice, and ratios of NP3/NP18-specific AFCs were plotted (C). (D) NIP binding B220− memory B cells were enumerated by flow cytometry. Each circle represents the result for an individual mouse. An asterisk indicates that the results are significantly different between TG and wt mice \((p < 0.05)\). The data are representative of two independent experiments.

(E–G) Splenocytes were recovered from either naïve or NP-CG-primed wt and TG mice at day 80 postimmunization or from CG-primed wt mice at days 50–70 postimmunization. Naïve or NP-CG-primed (Primed) B cells deprived of AFCs were purified by MACS and transferred into Rag-1−/− mice together with T cells purified from CG-primed wt mice. The recipient mice were bled at day 10 postchallenge, and the anti-NP IgG Ab titer was determined by ELISA with (E) NP3-BSA and (F) NP18-BSA as the capture antigen.

(G) Ratios of anti-NP3 IgG1 Ab versus anti-NP18 IgG1 Ab were plotted. The data are representative of two independent experiments.
Figure 5. Inhibition of Ras Activity Renders Memory B Cells Unresponsive to a Type II T Cell-Independent Antigen In Vitro

(A–D) Splenocytes were recovered from wt (open circles) and N-17-95 TG (closed circles) mice at day 60 postimmunization (A and B) or without immunization (C and D). After enrichment with MACS, NIP binding/B220⁺/IgG₁⁺/CD38⁺ cells (A and B) and NIP binding/B220⁺/IgM⁺/CD38⁺ cells (C and D) were sorted into 96-well plates at 300 cells/well. This was followed by cultivation for 6 days with mitomycin C treated 3T3 fibroblasts in the presence of a mixture of cytokines and either LPS (A and C) or NP-Ficoll (B and D). The amount of anti-NP₁₈ IgG₁ or IgM Ab in the culture supernatant was determined by ELISA. An asterisk indicates that the results are significantly different between TG and wt mice (p < 0.05). Data are representative of three independent experiments. Results are shown as mean ± SD of triplicate samples.

(E–H) NIP binding/B220⁺/IgG₁⁺/CD38⁺ cells (E and F) or NIP binding/B220⁺/IgM⁺/CD38⁺ cells (G and H) were sorted from NP-CG-primed wt or naïve mice, respectively. Sorted cells were cultured with either LPS (E and G) or NP-Ficoll (F and H) without (open circles) or with U0126 at 1.25 μM (closed triangles), 2.5 μM (closed squares), or 5 μM (closed circles). The amount of anti-NP₁₈ IgG₁ or IgM Ab in the culture supernatant was determined by ELISA. An asterisk indicates that the results are significantly different between TG and wt mice (p < 0.05). The data are representative of three independent experiments. Results are shown as mean ± SD of triplicate samples.

Response to recall antigen, most likely by promoting apoptosis. In addition, inhibition of the Ras cascade diminished the MZ B cell activation and/or differentiation into AFCs in response to T cell-dependent antigen.

We previously demonstrated that Ras deficiency reduces the life span of pre-B cells (Nagaoka et al., 2000), raising the idea that Ras deficiency might cause a generalized survival defect in all B cell subsets. However, several lines of evidence support the idea that the pro-survival effects of Ras signaling are not general but stage specific. For example, Ras is not required for the recruitment of dividing precursors into the mature B cell compartment and the survival of naive MZ and FO B cells (Figure S3A). In addition, Ras is not required for the primary antibody response by FO B cells (Figure 1) and the generation and proliferation of GC B cells in T cell-dependent response to NP-CG (Figure 2C and Figure S3B). Furthermore, Ras does not have a role in the persistence of memory B cells in a resting state after the primary response (Figure 2D and Figure S3B). Thus, antigen-stimulated memory and MZ B cells require the Ras cascade for their survival and/or differentiation whereas FO and GC B cells do not. Pre-B cells require the Ras cascade for their survival but naïve MZ and FO B cells do not. It has been suggested that BCR signaling mediates distinct effects in the immature and mature B cell development and activation (Niiro and Clark, 2002). We assume that the requirement of Ras activity in memory B cells is associated with their unique reactivity to antigen, as discussed below.

The inhibition of Ras activity caused a 10-fold reduction in the number of T1, T2, and T3 B cells, probably reflecting a developmental arrest at the transition from pro-B to pre-B cells (Nagaoka et al., 2000). However, we did not observe any effects on the accumulation and persistence of BrdU-labeled FO and MZ B cells in TG mice deficient in Ras activity, suggesting that Ras is dispensable for the generation of mature B cell compartments from dividing precursors and the survival of resting MZ and FO B cells. Thus, the reduced numbers of mature splenic B cells seem to largely reflect a developmental arrest in the BM rather than defective recruitment from transitional B cells or decreased survival of mature B cell compartments.

Adoptive transfer experiments suggest that Ras is required for the antigen-driven activation and terminal
Figure 6. Impaired IgG Antibody Production by Ras-Defective Memory B Cells Is Countered by Overexpression of Bcl-2

(A) NIP binding memory B cells were purified from the pooled spleens of wild-type mice (wt memory B cells) and N-17-95 TG mice (TG memory B cells). These were provided for cultivation stimulated without (medium) or with LPS or NP-Ficoll in the presence or absence of U0126. After 24 hr, the cultured cells were harvested and analyzed by RT-PCR with specific primers for Blimp-1 and the membrane or secretory form of IgG1. The signal intensity of the PCR products shown was quantified with the ImageGauge program version 4.1 (Fuji Photo Film). The data are representative of three independent experiments.

(B and C) B cells were purified from the pooled spleens of NP-CG-primed wt, N-17-95 TG (Ras), N-17-95 TG × Bcl-2 TG (Ras Bcl-2), and Bcl-2 TG (Bcl-2) mice at days 95–110 postimmunization. T cells were purified from CG-primed wt mice at days 50–70 postimmunization. The number of memory B cells was assessed by FACS prior to the transfer, and a mixture of B cells including 3000 memory cells and 1.5 × 10⁷ T cells was intravenously transferred into Rag-1−/− mice followed by challenge with 25 μg of NP-CG. The recipient mice were bled at day 10 postchallenge and then the anti-NP IgG1 Ab titer was determined by ELISA with NP3-BSA and NP18-BSA as the capture antigen (B). (C) Ratios of anti-NP3 IgG1 Ab versus anti-NP18 IgG1 Ab were plotted. An asterisk indicates that the results are significantly different between TG and wt mice (p < 0.05). The data are representative of two independent experiments.

(D and E) Splenocytes were recovered from wt (open circles), N-17-95 TG (closed circles), and N-17-95 TG mice that overexpressed Bcl-2 (closed squares) at day 60 postimmunization. Purified NIP binding/B220+/IgG1high/CD38+ cells were stimulated with LPS (D) or NP-Ficoll (E) as described in Figure 5. The amount of anti-NP IgG1 Ab in the culture supernatant was determined by ELISA. An asterisk indicates that the results are significantly different between TG and wt mice (p < 0.05). The data are representative of three independent experiments.

differentiation of MZ B cells to IgG AFC, but not FO B cells. The deficit in the MZ B cells for the primary response by inhibition of Ras activity may reflect their distinct properties that differ from FO B cells but that they share with memory B cells in several criteria, including lower activation threshold (Martin and Kearney, 2000). Thus, TG mice generate normal primary response to T cell-dependent antigen despite the reduction in the number of FO B cells and functional defects in MZ B cells. This leads to the idea that the antibody production of TG B cells is enhanced; however, the comparable primary antibody response in the mice reconstituted with an equal number of TG and wt B cells does not support this idea (Figures 1E and 1F). We speculate that the number of antigen-reactive B cells could generally be larger than the limit of the cells involved in the interaction with limited numbers of helper T cells at the primary response. Antigen-activated B cells generated in the response require the appropriate space for their persistence, i.e. MZ for memory B cells.
and BM for long-lived AFCs. Thus, the interaction with T cells and/or the subsequent competition for survival niches, rather than the number of antigen-reactive B cells, could be the strict limiting factor for the magnitude of T cell-dependent B cell response.

Ras-defective IgG memory B cells comprise a large fraction of low-affinity cells, probably owing to the impaired recruitment of high-affinity cells (Figures 3 and 4G, Table 1). We and others have proposed that IgG memory B cell development might not require the expression of high-affinity BCR or the GC environment (McHeyzer-Williams et al., 1991; Takahashi et al., 2001; Toyama et al., 2002), thus allowing the recruitment of low-affinity B cells without somatic mutations into the memory compartment during the early immune response. Such low-affinity memory B cells can mount secondary responses in adoptive hosts or in culture as efficiently as high-affinity memory B cells (Toyama et al., 2002; Inamine et al., 2005). However, Ras-defective memory B cells produced only small amounts of IgG Ab in response to secondary challenge. Furthermore, Ras-defective memory B cells failed to mount Ab responses when cultured with NP-Ficoll in vitro, although the same antigen triggered Ab production by wt memory B cells and Ras-defective naïve B cells. Therefore, the impaired terminal differentiation of Ras-defective memory B cells is likely attributable to a BCR signaling defect. That Ras is required for the transition of IgG memory B cells to AFCs was supported by our demonstration that MEK inhibition impaired in vitro Ab responses of wt IgG memory B cells, but not naïve IgM B cells. These data indicate that BCR signaling through the Ras-Raf-MEK cascade is required for the terminal differentiation of IgG memory B cells into Ig-secretory plasma cells.

We observed that overexpression of BCl-2 restored the IgG Ab response of Ras-defective memory B cells stimulated by NP-CG in vivo and with NP-Ficoll in vitro. IgG memory B cells could therefore be susceptible to apoptosis upon BCR stimulation, a situation normally countered by the antiapoptotic effects of the Ras cascade. It is unclear why BCR engagement causes apoptosis in memory, but not in naïve B cells, when the Ras cascade is inhibited. It has been suggested that the IgG BCR complex delivers a stronger signal than the IgM BCR (Martin and Goodnow, 2002). Similarly, Feldhahn et al. (2002) have predicted that memory B cells amplify BCR signals by increasing the levels of positive regulators and reducing the negative regulators. Adaptations that increase BCR signal strength in memory B cells could be advantageous for prompt responses to a minute amount of antigen. However, hyperreactivity to antigen may increase the susceptibility to apoptosis, as observed by the hypercrosslinking of the BCR on mature B cells that induces apoptosis in vitro (Berard et al., 1999). Hyperreactivity of memory B cells to antigen might be augmented further in high-affinity cells, which may, in turn, result in the preferential loss of B cells carrying an affinity-enhancing mutation in the Ras-defective memory compartment (Figure 3 and Table 1).

We observed that Ras deficiency did not affect the selection for high-affinity B cells in GC and their recruitment into AFCs; however, it impaired the recruitment of high-affinity cells into the memory compartment. These results imply that the recruitment process of high-affinity cells into the memory or AFC compartment could be independently regulated in the post-GC phase (Smith et al., 2000; Takahashi et al., 2001). One possible explanation for the independent recruitment would be the differential regulation of sIg expression on these two populations; clonally selected GC B cells upregulate sIg at the development process into memory B cells, whereas those for AFCs are downregulated. In this scenario, memory B cell precursors are likely to increase the susceptibility to BCR-induced cell death at the transition to memory B cells, but not to AFCs. Further studies are needed to clarify this issue.

A point of interest is how the Ras cascade supports memory B cell survival during terminal differentiation and antigen-driven selection. Pharmacological inhibition of MEK activity impaired the terminal differentiation of memory B cells, supporting the notion that the Ras-Raf-MEK pathway in BCR signaling inhibits memory B cell apoptosis during plasmacytic differentiation. BCR crosslinking promotes interaction of Bim with Bcl-2 (Enders et al., 2003), thereby releasing the proapoptotic proteins Bax or Bak (Cory and Adams, 2002). The Ras-Raf-MEK pathway interferes with apoptosis by phosphorylating Bim, leading to its degradation via the proteasome pathway (Ley et al., 2003). In addition, the Ras pathway interferes with apoptosis at the level of cytosolic caspase activation (Allan et al., 2003). However, stimulation with NP-Ficoll in the presence of caspase inhibitors did not improve the response of Ras-defective memory B cells (data not shown), thereby raising the view that BCR activation in Ras-defective memory B cells initiates apoptosis by a caspase-independent pathway. Further analysis will be required to identify the mechanisms of BCR-induced apoptosis in memory B cells.

In summary, our studies shed new light on the cellular and molecular basis of the memory B cell response. Memory B cells exhibit a unique state of antigen hyperreactivity and susceptibility to apoptosis that relates, at least in part, to BCR signal strength. The proapoptotic consequence of strong BCR signaling is countered by the antiapoptotic effect of the Ras cascade. A strong signal by the IgG BCR complex on memory B cells might result from the expression level of BCR on the surface in combination with the intrinsic signaling activity that might qualitatively and/or quantitatively differ from other developmental stages of B cells. In this context, we have been searching for the cell-signaling molecules that are up- or downregulated in memory B cells. Further analysis is needed to clarify how this signaling capacity of memory B cells is unique.

Experimental Procedures

Animals and Immunization

Specific pathogen-free (SPF) female C57BL/6 mice were purchased from Japan SLC Inc. (Shizuoka, Japan). C57BL/6-Rag1−/− and C57BL/6-scid mice were purchased from the Jackson Laboratory (Bar Harbor, MA). C57BL/6-bcl2 transgenic mice were provided by Dr. Tsubata (Tokyo Medical and Dental University). Transgenic mice expressing a dominant inhibitory mutant of Asn-17 Ha-ras were established as reported previously (Nagaoka et al., 2000). Two
independent transgenic lines designated N-17-95 and N-17-52 were used as the homozygous mice. Female mice aged 12–15 weeks were intraperitoneally injected with 100 µg of NPs-CG conjugate or CG precipitated in alum. 25 or 50 micrograms of soluble NPs-CG was intravenously administered for the secondary challenge. All mice were used in accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases, Japan.

Cell-Surface Staining and FACS Analysis
Cell staining was carried out as previously described (Takahashi et al., 2001). Briefly, cells were pretreated with anti-FcγRII/III mAb (2.4G2; BD Biosciences, San Jose, CA) and then incubated with biotinylated mAbs against IgM, IgD, CD43, CD8, Gr-1, and CD11b (BD Biosciences). This was followed by staining with AlexaFluor 647-coupled anti-CD38, AlexaFluor 594-coupled anti-CD220, FITC-coupled anti-IgG1 (BD Biosciences), and PE-conjugated NIP-BSA. To detect plasma memory B cells, anti-CD43 and CD11b mAbs were excluded from the mixture of biotinylated mAbs. To purify NPs-specific GC and memory B cells, IgM-IgD-CD43-CD8−CD11b− splenocytes were enriched with a MACS column (Miltenyi Biotec, Gladbach, Germany) followed by staining with fluorescence-conjugated reagents. After washing, cells were incubated with Tricolor-conjugated streptavidin and resuspended in a staining buffer containing propidium iodide. Stained cells were analyzed or purified using a FACS Vantage (BD Biosciences) as described previously (Takahashi et al., 2001).

ELISA and ELISPOT Assays
ELISA and ELISPOT assays were performed with NPs-BSA and NPs×-BSA, as described previously (Takahashi et al., 1998). The relative affinity of anti-NP Abs was estimated by calculating the ratio of anti-NP/anti-NP× Ab.

Sequence Analysis of V<sublambda></sub> Genes
NIP binding GC and memory B cells were sorted directly into proteinase K solution and processed as described previously (Takahashi et al., 2001). BM AFCs were sorted directly into Trizol (Invitrogen, Frederick, MD) containing 5 µg/ml tRNA (Roche Diagnostics, Mannheim, Germany). V<sublambda></sub> gene cDNAs were amplified by PCR and the nucleotide sequences of the cloned V<sublambda></sub> genes were determined as described previously (Takahashi et al., 1998).

Adoptive-Transfer Experiment
B cells and T cells were purified from the pooled spleens of naive or immunized mice by using a MACS system with biotinylated mAbs against AA4.1, CD43, CD138, CD3, CD4, CD8, Gr-1, and CD11b (B cells); AA4.1, CD43, CD138, CD3, CD8, Gr-1, CD11b, CD23, and Fas (MZ B cells); B220, IgM, IgD, NK1.1, Gr-1, and CD11b (T cells); B220, IgM, IgD, NK1.1, Gr-1, CD11b, and CD6 (CD4 T cells). For FO B cells, a CD23<sup>+</sup> fraction was further collected by using PE-conjugated anti-CD23 (BD Biosciences) followed by anti-PE beads (Miltenyi Biotec). The procedure yielded >95% purity for B cells and FO B cells, >90% purity for T cells and CD4 T cells, and >90% purity for MZ B cells. Purified B cells (3 × 10<sup>5</sup>/head) together with T cells (1.5 × 10<sup>6</sup>/head) or purified FO/MZ B cells (FO, 3 × 10<sup>5</sup>/head; MZ, 9 × 10<sup>5</sup>/head) together with CD4 T cells (4 × 10<sup>6</sup>/head) were intravenously injected into C57BL/6 Rag-1<sup>−/−</sup> mice or C57BL/6-scid mice (Song and Cerny, 2003), respectively. When Bcl-2 TG mice were used as a source of B cells, NIP binding IgG<subμ</sub> memory B cells included in the purified B cells were enumerated by flow cytometry, and the number of transferred B cells was adjusted to contain an equal number of memory B cells as those from wt mice (3 × 10<sup>5</sup> cells). The recipient mice were challenged with either soluble NP-CG or NPs-CG in alum 12 hr later.

Cell Culture
One day before sorting, mitomycin C-treated BALB/c 3T3 fibroblasts were dispensed into 96-well, flat-bottom plates at 10<sup>4</sup> cells per well. NIP binding IgG<subμ</sub> memory B cells or NIP binding naïve B cells (B220<sup⁺</sup>IgM<sup⁻</sup>IgD<sup⁻</sup>CD3<sup⁺</sup>) were sorted from NP-CG-primed mice or naïve mice, respectively, and then deposited at 300 cells/well by using cloning cell (BD Biosciences). Sorted cells were cultured for 6 days in Dulbecco's modified Eagle's medium (Sigma, St. Louis, MO) supplemented with 20% FCS, 5 × 10<sup⁻⁴</sup>M 2-ME (Invitrogen), 1 mM sodium pyruvate (Invitrogen), 10 mM HEPES (Invitrogen), 0.1 mM nonessential amino acid (Invitrogen), 100 U/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen), 20 U/ml rIL-2 (Sigma), 400 U/ml rIL-4 (Pepro Tech, London, UK), and 50 U/ml rIL-5 (Genzyme-Technie, Minneapolis, MN). Cells were stimulated with 40 µg/ml LPS (Escherichia coli, 0111:B4, Sigma) and 10 ng/ml NPs×-Ficoll (Biosearch Technologies, Novato, CA) for the first 24 hr of culture. MEK inhibitor U0126 (Calbiochem, Schwabach, Germany) was also added to the culture medium when indicated. The culture medium was exchanged every day, and the amount of anti-NP IgM and IgG<subμ</sub> Ab in the supernatant was determined by ELISA.

RT-PCR Analysis
Total RNA was extracted from Trizol-solubilized cells according to the manufacturer's protocol. First-strand cDNA was synthesized from DNase I-treated total RNA by using oligo d(T) primer and a Superscript II Kit (Invitrogen). The cDNAs were amplified by PCR with the following conditions: denaturation at 94°C for 30 s, annealing at 58°C for 20 s, and extension at 72°C for 60 s. A total of 40 cycles were performed for the detection of Blimp-1 cDNA and 37 cycles for IgG<subμ</sub>. The oligonucleotide primers were: Blimp-1 sense GAGGGAAAATTTGAGAGTAGCT-3<sup#</sup> and Blimp-1 antisense 5'-CACATGAGTGCCAGGTCTGC-3'. The amplicons were separated by 1.5% agarose gel electrophoresis and stained with ethidium bromide. The signal intensity of the PCR products was quantified with the ImageGauge program version 4.1 (Fuji Photo Film, Tokyo, Japan).

Statistical Analysis
Fisher's exact test, the Mann-Whitney nonparametric (two tailed, n > 4) test, and the Student's t test (two tailed, n = 3) were used within the StatView program (Abacus Concepts, Inc., Cary, NC). A probability of p < 0.05 was considered to indicate a statistically significant difference.

Supplemental Data
Supplemental Data including five figures are available online with this article at http://www.immunity.com/cgi/content/full/23/2/127/ DC1/.

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References


**Accession Numbers**

Complete sequence data are available from the DNA Data Bank of Japan (DDBJ), the EMBL Nucleotide Sequence Database, and GenBank under the following accession numbers: GC, AB121447–AB121493; memory, AB121494–AB121621; and AFC, AB121622–AB121664.