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*J Immunol* published online 13 April 2011
http://www.jimmunol.org/content/early/2011/04/12/jimmunol.1100213

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**Supplementary Material**

http://www.jimmunol.org/content/suppl/2011/04/12/jimmunol.1100213.DC1

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Tolerance Induction of IgG+ Memory B Cells by T Cell-Independent Type II Antigens

Kei Haniuda, Takuya Nojima, Kyosuke Ohyama, and Daisuke Kitamura

Memory B cells generated during a T cell-dependent immune response rapidly respond to a secondary immunization by producing abundant IgG Abs that bind cognate Ag with high affinity. It is currently unclear whether this heightened recall response by memory B cells is due to augmented IgG-BCR signaling, which has only been demonstrated in the context of naive transgenic B cells. To address this question, we examined whether memory B cells can respond in vivo to Ags that stimulate only through BCR, namely T cell-independent type II (TI-II) Ags. In this study, we show that the TI-II Ag (4-hydroxy-3-nitrophenyl) acetyl (NP)-Ficoll cannot elicit the recall response in mice first immunized with the T cell-dependent Ag NP-chicken γ-globulin. Moreover, the NP-Ficoll challenge in vivo as well as in vitro significantly inhibits a subsequent recall response to NP-chicken γ-globulin in a B cell-intrinsic manner. This NP-Ficoll-mediated tolerance is caused by the preferential elimination of IgG+ memory B cells binding to NP with high affinity. These data indicate that BCR cross-linking with a TI-II Ag does not activate IgG+ memory B cells, but rather tolerizes them, identifying a terminal checkpoint of memory B cell differentiation that may prevent autoimmunity. The Journal of Immunology, 2011, 186: 000–000.

The heightened memory recall response is believed to be attributable to both an increase in the frequency of Ag-specific memory B and T cells and the enhanced capacity of the B cells for expansion and facilitated differentiation into plasma cells when compared with naive B cells. These latter features can be ascribed in part to enhanced engagement of Th cells by the memory B cells due to their expression of higher levels of MHC class II, CD80, and other T cell-interacting molecules (10–13). Alternatively or additionally, the phenotype of memory B cells can be ascribed to augmented signaling through the IgG BCR that is expressed by most of these cells. Indeed, increased B cell clonal expansion, plasma cell production, and Ab production upon primary immunization were observed in mice transgenic for an IgG BCR or a chimeric BCR consisting of the extracellular portion of IgM and the transmembrane/cytoplasmic domains of IgG (IgM/G) compared with mice expressing an IgM BCR (14). Furthermore, the IgG BCR, or its cytoplasmic domain, has been shown to mediate augmented calcium response, BCR oligomerization, microcluster growth, and proliferation upon BCR ligation (15–19). However, these results have been obtained from studies using transgenic naïve B cells or B cell lines and not naturally derived memory B cells expressing an IgG BCR. Thus, there is no formal evidence demonstrating that the enhanced response of memory B cells is due to enhanced signaling through IgG BCR.

This issue would be best addressed by stimulating memory B cells in vivo only through the BCR without engaging T cell help. T cell-independent (TI) Ags are known to induce a primary immune response in the absence of T cell help, characterized by the rapid production of low-affinity Abs of IgM as well as particular IgG subclasses, mainly IgG3 in mice (20). Among the TI Ags, T cell-independent type I (TI-I) Ags contain mitogenic moieties such as LPS and activate B cells through the BCR and TLRs simultaneously. By contrast, TI type II (TI-II) Ags are large molecules that display highly repetitive epitopes on their surface and can directly stimulate B cells by cross-linking the BCR without engagement of other known costimulatory receptors. If the heightened memory B cell response is due to heightened IgG-BCR signaling, then TI-II Ags carrying the same epitope as the priming TD Ag should elicit a recall response, inducing much stronger
activation and more Ab production by IgG⁺ memory B cells than by naive IgM⁺ B cells. Surprisingly, this seemingly old and important question has remained unsolved. Experimental data that can be found only in the classical literature had drawn two opposite conclusions: TI-II Ags can, or cannot, elicit the recall response (21–24).

In this study, we have used current technology to address whether TD-induced IgG⁺ memory B cells respond to epitope-matched TI Ags. Our data clearly indicate that IgG⁺ memory B cells are activated by a TI-I Ag containing LPS, but not by a TI-II Ag. Moreover, we found that memory B cells with a BCR of high affinity for the antigenic epitope are deleted upon challenge with the TI-II Ag by B cell-intrinsic mechanisms. As a result, a subsequent recall response to the TD version of the Ag was significantly reduced, consisting only of low-affinity Abs. These data indicate that BCR cross-linking with a TI-II Ag does not activate IgG⁺ memory B cells, but rather tolerizes them, suggesting the presence of a terminal checkpoint in B cell differentiation that may help prevent autoimmunity.

Materials and Methods

Mice and immunizations

C57BL/6 mice were purchased from Japan SLC. B1-8 IgH knockin (B1-8 ki) mice (25) backcrossed to the congenic C57BL/6-CD45.1 strains were used where indicated. Previous studies have demonstrated that the B1-8 ki allele effectively excludes the endogenous IgH allele (26). Therefore, we used heterozygous mice in all experiments. All mice were maintained in a mouse facility under specific pathogen-free conditions. Mouse procedures were performed under the protocols approved by the Animal Care and Use Committee of the Tokyo University of Science. Mice were immunized i.p. with 100 μg (4-hydroxy-3-nitrophenyl) acetyl (NP)₃-chicken γ-globulin (CGG) precipitated in aluminum hydroxide (alum) and boosted i.v. with 25 μg soluble NP3-CGG, NP3₁₀-LPS (Biosearch Technologies), NP₁₀-Ficoll (Biosearch Technologies), and NP₁₀-mouse serum albumin (MSA) in PBS, unless otherwise noted.

Immunofluorescence microscopy

Freshly isolated spleens were snap-frozen in OCT compound (Sakura Finetek) and 5 to 6-μm-thick sections were prepared using a cryostat. After drying at room temperature, sections were fixed for 10 min in acetone at −20°C and allowed to dry. Nonspecific binding was blocked using a 3% BSA/PBS for 30 min at room temperature, and then the slides were washed and stained for 1 h at room temperature with different combinations of the following Abs and reagents: PE-conjugated anti-IgG1 (Southern Biotechnology Associates), biotinylated anti-CD45R/B220, FITC-conjugated anti-GL7, CD45R/B220 (eBioscience), 7-amino-4-methylcoumarin-3-acetic acid–conjugated streptavidin (Jackson ImmunoResearch Laboratories), PE-conjugated streptavidin (eBioscience), and biotinylated NP⁺-BASA. After washing, slides were mounted with Fluorescent Mounting medium (DakoCytomation). The samples were examined by an immunofluorescence microscope (BZ-9000; KEYENCE).

Flow cytometry

Single-cell suspensions from spleens were prepared at the indicated days postimmunization. RBCs were lysed with ammonium chloride. Cells were incubated with anti-FcγRII/III Ab (2.4G2) to block FcγRs and were stained for 30 min on ice in PBS containing 0.5% EDTA and 0.05% sodium azide with different combinations of the following Abs and reagents conjugated with FITC, PE, biotin, PE-Cy7, or allophycocyanin: anti-IgG1 (Southern Biotechnology Associates), anti-CD45.1 (Beckman Coulter), anti-CD19, CD38, CD45R/B220, CD80, CD138, GL7 (eBioscience), anti-CD95 (BD Biosciences), streptavidin (eBioscience), and biotinylated NP⁺-BASA. For Annexin V staining, cells were incubated with FITC-conjugated Annexin V (MBL International) on ice for 10 min. All samples were analyzed using an FACSCalibur flow cytometer or FACS Canto II (BD Biosciences). The data were analyzed using FlowJo (Tree Star).

Adoptive transfer

Total spleen cells were collected from NP-CGG/alum-immunized or PBS-injected C57BL/6 mice. RBCs were lysed with ammonium chloride, and 2 × 10⁷ cells were transferred i.v. into nonimmunized C57BL/6 mice 24 h before secondary immunization with soluble Ags. For B cell transfer, non-B cells were depleted from spleen cells of B1-8 ki CD45.1 mice by incubation with biotinylated Abs against CD43, CD4, CD8, DX5, and Ter-119 (eBioscience) followed by negative sorting using the MACS system (Miltenyi Biotec) and iMag system (BD Biosciences). The procedure yielded >98% purity of B cells. Nonimmunized C57BL/6-CD45.2 mice were transferred i.v. with 2 × 10⁷ isolated B cells 24 h before the secondary immunization.

BrdU labeling and detection

For BrdU labeling, mice were given i.p. injections of 0.6 mg BrdU (Sigma-Aldrich) every 12 h from 10–14 d post-primary immunization and were sacrificed at the indicated time points. Single-cell suspensions were stained for surface markers as described above, then washed with cold PBS, resuspended in 0.5 ml cold 0.15 M NaCl, to which 1.2 ml cold 95% ethanol was added dropwise while the tube was gently vortexed, and kept 30 min on ice. Cells were washed and fixed with 1 ml 1% parafomaldehyde/PBS containing 0.05% Tween 20 overnight at 4°C. Fixed cells were resuspended in 1 ml 0.15 M NaCl, 4.2 mM MgCl₂, and 10 μM HCl containing 30 U/ml DNase I (Sigma-Aldrich) and incubated for 30 min at room temperature. After being washed twice with PBS, cells were stained overnight at 4°C with FITC-conjugated anti-BrdU (BD Biosciences) and analyzed with FACS Canto II (BD Biosciences).

ELISA and ELISPOT assays

NP-specific Ab-forming cells (AFCs) were detected by ELISPOT assay using the MultiScreen 96-well filtration plate (Millipore) coated with 10 μg/ml NP₀₉-BSA or NP₁₁-BSA for total or high-affinity anti-NP AFC, respectively. Serially diluted cells were added into individual wells in triplicate, then incubated for 6 h in a humidified atmosphere at 37°C with 5% CO₂. Anti-NP IgG1 spots were revealed by HRP-conjugated goat anti-mouse IgG1 Ab (Southern Biotechnology Associates) in conjunction with AEC substrate (DakoCytomation). ELISA was performed as follows: flat-bottom 96-well plates (Nunc) were coated with 10 μg/ml NP₀₉-BSA or NP₁₁-BSA, blocked with 3% FCS, and then serially diluted serum samples were added to individual wells. Bound Abs were revealed by HRP-conjugated goat anti-mouse IgG1 Ab in conjunction with TMB substrate (Sigma-Aldrich). Absorbance at 450 nm was measured with a microplate reader (Benchmark; Bio-Rad).

RT–PCR and nucleotide sequence analysis

RT-PCR and nucleotide sequence assays were performed as described previously (27).

Statistical analysis

Statistical analysis was performed using the Student t test.

Results

TI-I but not TI-II Ags induce a memory recall response

To analyze whether IgG⁺ memory B cells can respond to TI Ags and differentiate to AFCs, we first immunized C57BL/6 mice with a commonly used TD Ag, NP-CGG precipitated in alum and, 16 wk later, challenged the mice with TD, TI-I, or TI-II Ags carrying the same epitopes, NP-CGG, NP-LPS, or NP-Ficoll, respectively. The mice rechallenged with NP-CGG rapidly produced a robust NP-specific IgG1 response, as measured by serum Ab and splenic AFCs, as early as 5 d postimmunization, whereas those challenged with NP-LPS had a rapid but more modest response. In striking contrast, mice secondarily challenged with NP-Ficoll had no significant NP-specific IgG1 response (Fig. 1A, 1B).

The response of mice primed with NP-CGG and then challenged with NP-LPS is an authentic memory recall response because: 1) primary immunization with NP-LPS did not result in production of NP-specific IgG1⁺ AFCs in 5 d (Fig. 1B); and 2) the ratio of the number of high-affinity NP-AFCs to total NP-AFCs was equally high after the secondary challenge with NP-LPS or NP-CGG (Fig. 1C). Challenge with LPS alone did not elicit the IgG1 recall response (Fig. 1D), in agreement with recent reports using similar systems (28, 29).
It was previously reported that pre-existing Ag-specific IgG Abs strongly suppress Ag-specific IgM production from TI memory B cells upon a secondary challenge with a TI-II Ag (30). To exclude the possibility that the inability of NP-Ficoll to elicit a specific IgG1 recall response is due to pre-existing NP-specific Abs, we transferred splenocytes from mice primed with NP-CGG 112 d earlier into naive recipients, which were then challenged with NP-Ficoll or NP-CGG (Fig. 1E). Even in this condition, NP-Ficoll did not induce a specific IgG1 recall response (Fig. 1E). These data indicate that the inability of the TI-II Ag to recall the memory response is not due to pre-existing Ag-specific Abs, but is attributable to postimmune cellular components.

**TD and TI-I, but not TI-II, challenge induces a rapid extrafollicular foci response**

To confirm the above data in situ, NP-CGG–primed mice were challenged with soluble NP-CGG, NP-LPS, or NP-Ficoll 16 wk later, and then spleen sections were analyzed by immunofluorescence microscopy. In the primary response, IgG1+ cells emerged by day 5, sparsely scattered in the follicles. There were a few small clusters of GL7+IgG1+ cells near the T cell zones, which are presumably immature GCs (Fig. 2B). By day 10, many IgG1+ cells were found in GCs, and some were seen in foci in the red pulp (Fig. 2C). By contrast, large numbers of IgG1+ cells emerged in the follicles at day 2 after the secondary immunization with NP-CGG, and they moved to the red pulp and formed large foci of AFCs by day 5, which declined in size by day 10 (Fig. 2D–F). A few GCs were detectable at day 10, but they were not NP specific (data not shown). These data indicate that IgG1+ memory B cells swiftly expand in the follicles in response to rechallenge with a TD Ag and differentiate into extrafollicular AFCs, which are much more numerous than in the primary response. In addition, the NP-specific IgG1+ memory B cells rarely generate new GCs.

Secondary challenge with NP-LPS also induced the foci of AFCs by day 5, but to a lesser extent than with NP-CGG (Fig. 2G, 2H). By contrast, essentially no IgG1+ AFCs (Fig. 2J–L) or anti-NP AFCs of any isotype (Fig. 2M) were detectable in the sections from mice secondarily challenged with NP-Ficoll, contrasting to the robust anti-NP extrafollicular foci formation after primary immunization with NP-Ficoll (Fig. 2N). Small GC-like clusters in the follicles were observed in the sections from all the mice primed with NP-CGG irrespective of the secondary challenge. These data indicate that NP-specific IgG1+ memory B cells do not differentiate into AFCs upon encounter with multivalent NP-Ags that do not elicit T cell help, except when a TLR is costimulated.

**TI-II Ag tolerizes IgG1+ memory B cells binding Ag with high affinity through a B cell-intrinsic manner**

We next asked whether the Ag-specific memory B cells simply ignore the TI-II Ags or recognize them and are somehow affected. For this, we challenged NP-CGG–primed mice with NP-Ficoll or PBS as a control at day 60, and then challenged the mice again with soluble NP-CGG 3 wk later. Upon tertiary challenge with NP-CGG, the anti-NP IgG1 response was significantly lower in mice that had been secondarily challenged with NP-Ficoll as compared with mock (PBS)-challenged mice (Fig. 3A). This effect was due mainly to the loss of IgG1 Abs that bind NP with relatively high affinity (Fig. 3B, 3C). This serological finding was
directly confirmed by sequencing γ1 transcripts containing V₁H186.2 and related H chain V region (V₁H) sequences from sorted splenic plasma cells (CD4⁻ CD8⁻ k⁻ B220lowCD138⁺) induced by the tertiary challenge with NP-CGG. NP responses in C57BL/6 mice are known to be dominated by Abs comprised of a VH186.2 H chain and L chain, and the H chains of high-affinity Abs carry a replacement mutation, tryptophan to leucine at codon 33 (W33L), which results in a 10-fold increase in the affinity of the Ab for NP (31). All 12 clones obtained from mock-challenged mice used VH186.2, and 66.7% of these contained the W33L replacement. By contrast, only 2 out of 12 clones obtained from the NP-Ficoll–challenged mice used VH186.2, and none of the sequences had the W33L replacement (Table I).

To determine whether the defect in the recall response is B cell intrinsic, purified splenic B cells from mice that had been primed with NP-CGG and challenged with various doses of NP-Ficoll were transferred with 10⁷ CGG-primed splenocytes (primed T cell) into naive recipients, which were then immunized and analyzed exactly as in D. n = 3 per group. One of two experiments with similar results is shown.
3 wk before were transferred with CGG-primed splenocytes into recipient mice. Upon a tertiary challenge with NP-CGG, the recipients of NP-Ficoll–challenged B cells did not produce NP-specific IgG1 Ab, whereas recipients of mock-challenged B cells responded well (Fig. 3D). We next treated splenic B cells from the NP-CGG–primed mice with NP-Ficoll in vitro, then transferred them into recipient mice with CGG-primed T cells, and then challenged with NP-CGG (Fig. 3E). Compared with the mice receiving untreated B cells, those receiving NP-Ficoll–treated B cells produced much less anti-NP IgG1 Ab in a manner depending on the dose of NP-Ficoll. Furthermore, this NP-Ficoll–mediated suppression was not reversed by simultaneous treatment with Th cytokines such as IL-2, IL-4, and IL-5 (Fig. 3F). These results indicate that Ag-specific high-affinity IgG1 memory B cells are tolerized by TI-II Ag through a B cell-intrinsic mechanism. Ag-specific memory B cells do not expand or differentiate into AFCs, but are eliminated in response to TI-II Ags.

To determine how TI-II Ags induce tolerance of the Ag-specific memory B cells at a cellular level, we adopted an established B cell transfer system using V_{H}1-B1-8 gene knockin (B1-8) mice. In these mice, a small percentage of peripheral B cells, which are almost exclusively λk, are known to bind NP. Small numbers of splenic B cells from B1-8 mice congenic for the CD45.1 allelic marker were transferred into naive C57BL/6 mice (CD45.2), and splenic B cells from B1-8 ki mice congenic for the CD45.1 allelic marker were transferred into naive C57BL/6 mice (CD45.2). The data shown in Figs. 3 and 4 could also be interpreted as high-affinity binding by TI-II Ags inducing clonal deletion of memory B cells that have generated in the late phase of GC reaction at least partly by apoptosis.

TI-II Ag challenge eliminates IgG1 memory B cells selected for high-affinity Ag binding

The data shown in Figs. 3 and 4 could also be interpreted as showing that NP^{high} memory B cells were anergized, rather than deleted, upon binding to NP-Ficoll through downmodulation of their BCR. If this were the case, such memory B cells would be hidden in the donor-derived NP^{high} population after the NP-Ficoll challenge in the system described in Fig. 4, but they must retain the normal frequency of V_{H}168.2 genes encoding the H chain binding to NP with high affinity. To evaluate this possibility, we sorted all donor-derived B cells except plasma and GC B cells (CD45.1^{+}B220^{+}CD138^{+}CD38^{low}) from the spleens of the recipient mice 5 d after the secondary challenge with NP-Ficoll or PBS as described in Fig. 4 and sequenced the V_{H}168.2-C_{H}1 transcripts from these cells. The sequences from both groups of mice were fully mutated, indicating that they were derived from NP-specific plasma cells in the bone marrow as assessed by ELISPOT assays (data not shown). The outcome in the mice challenged with NP-Ficoll was very different: the NP^{high} memory B cell population did not expand but rather contracted, and the NP^{low} population remained unchanged (Fig. 4A and data not shown). The NP^{high} cells did not have an activated phenotype, except for an increased cell size (Fig. 4B), and there was no burst of donor-derived plasma cells (Fig. 4C). The NP-Ficoll challenge appeared to induce selective elimination of NP-specific memory B cells and was sensitive to small amounts of the Ag (Fig. 4D). As a control, primary immunization with NP-Ficoll induced a marked expansion of donor-derived naive NP^{+} B cells in recipient mice by day 10 (Fig. 4E).

Next we analyzed the BrdU content of the NP^{high} memory B cells on day 5 after the secondary challenge. Whereas 5.5% of such cells retained BrdU in the control mice, <1% retained BrdU in the NP-CGG–challenged mice, and essentially no cells were BrdU^{+} in NP-Ficoll–challenged mice (Fig. 4F). The loss of BrdU^{+} cells is likely due to the rapid proliferation of these cells in the NP-CGG–challenged mice, but to an elimination of such cells in the NP-Ficoll–challenged mice, considering the results shown in Fig. 4A and 4D. This interpretation is also supported by an independent experiment showing that the NP^{high} memory B cells began to undergo apoptosis on day 2 in NP-Ficoll–challenged mice, before the NP^{high} cells began to expand in the NP-CGG–challenged mice (Supplemental Fig. 2). Together, these data strongly suggest that high-affinity binding by TI-II Ags induces clonal deletion of memory B cells that have generated in the late phase of GC reaction at least partly by apoptosis.

### Table I. Secondary NP-Ficoll challenge affects anti-NP V_{H} gene repertoire and SMH pattern in splenic plasma cells after tertiary NP-CGG challenge

<table>
<thead>
<tr>
<th>V_{H}186.2</th>
<th>Non-V_{H}186.2</th>
<th>V_{H} Mutation(^{a}) Average</th>
<th>R/S Ratio(^{b})</th>
<th>Position 33(^{c})</th>
<th>R/S Ratio(^{d})</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS (n = 3)</td>
<td>12</td>
<td>0</td>
<td>11.6</td>
<td>6.3 (4.2/0.7)</td>
<td>11.1 (3.2/2.9)</td>
</tr>
<tr>
<td>NP-Ficoll (n = 3)</td>
<td>2</td>
<td>10</td>
<td>8.8</td>
<td>4.0 (2.7/0.7)</td>
<td>1.0 (2.7/2.6)</td>
</tr>
</tbody>
</table>

C57BL/6 mice were primed with NP-CGG/alum 60 d before and challenged with NP-Ficoll or PBS. Three weeks postchallenge, these mice were challenged with soluble NP-CGG. On day 5 after the tertiary challenge, splenic plasma cells (propidium iodide `− CD4 CD8^{−}CD20^{+}CD138^{+}CD19^{−}CD38^{−}Fas^{−}GL7^{−}W33L^{−}) were sorted from pooled splenocytes of these mice, from which IgH^{+} mRNAs containing V_{H}186.2 and related VH regions were sequenced.\(^{a}\) The average number of mutations per V_{H} sequence.\(^{b}\) The average numbers of replacement and silent mutations per V_{H} sequence are in parentheses.\(^{c}\) The percentage of clones carrying tryptophan-to-lysine replacement at codon position 33.
replacement/silent mutations (R/S ratio) in CDR1 and CDR2 was significantly lower in the former population (Table II and data not shown). These data indicate that IgG1+ memory B cells highly selected for high-affinity binding to NP were clonally deleted upon encounter with NP-Ficoll.

Tolerance of IgG1+ memory B cells is not induced by a TD Ag that is not recognized by T cells

Finally, we asked whether the deletion of Ag-specific IgG1+ memory B cells upon binding TI-II Ags is simply due to the lack of cognate T cell help. Thus, we challenged the NP-CGG–primed memory B cells (Fig. 4). IgG1+ memory B cells do not expand or differentiate into plasma cells, but are eliminated upon binding with a TI-II Ag. A–C and F, C57BL/6 (CD45.2) mice were adoptively transferred with $2 \times 10^5$ naive B1-8 ki CD45.1 B cells and immunized with NP-CGG/alum. BrdU was administered from days 10–14 after primary immunization. On day 60 postimmunization, the mice were injected with the indicated soluble Ags or PBS, and on the indicated days after immunization, pooled splenocytes from three to four mice per group were analyzed by flow cytometry. A, Profiles of CD45.1 expression versus NP binding in pooled splenocytes from mice challenged with NP-CGG (three mice), NP-Ficoll (four mice), or PBS (three mice). The plots are gated on live lymphocytes. Donor-derived (CD45.1+) NP$^{\text{high}}$ or NP$^{\text{low}}$ fractions are boxed. The numbers indicate percentages of total lymphocytes. B, Expression of the indicated cell-surface markers on the CD45.1+ NP$^{\text{high}}$ fractions from NP-CGG– or NP-Ficoll–challenged mice and control (PBS) mice (shown in A, day 5) are shown as histograms in comparison with B220$IgD^+$ splenic B cells taken from naive C57BL/6 mice (filled dark histograms). C, Profiles of CD45.1+ versus CD138 expression in the same samples as in A, day 5. D, Adoptive transfer, priming, the secondary challenge, and analysis were performed as in A, except that the mice were challenged i.p. with the indicated dose of NP-Ficoll 5 d before analysis. The plots are gated on live lymphocytes (top panel) or on the CD45.1+ CD38$^{\text{high}}$ populations (boxed on top panels) (middle panel). The numbers indicate percentage ± SD of each gated population. The actual number of CD45.1+CD38$^{\text{high}}$IgG1+NP$^+$ memory B cells in each mouse is plotted (bottom panel; n = 4 per group). The bar represents the mean in each group. E, Adoptive transfer was performed as in A, and the mice were immunized with NP-Ficoll 60 d later. On the indicated days after the immunization, pooled spleen cells from these mice (three mice per group) were analyzed. F, Intranuclear BrdU staining of the cells in CD45.1+NPhigh fractions in the same samples as in A, day 5. Data are representative of three independent experiments.

Table II. TI-II Ag induces tolerance of high-affinity IgG1+ memory B cells

<table>
<thead>
<tr>
<th>R/S Ratio $^a$</th>
<th>PBS (n = 3)</th>
<th>NP-Ficoll (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of Clones Sequenced</td>
<td>12</td>
<td>15</td>
</tr>
<tr>
<td>$V_H$ Mutation$^a$</td>
<td>9.4</td>
<td>7.9</td>
</tr>
<tr>
<td>CDR1+2</td>
<td>6.1 (4.1/0.7)</td>
<td>3.0 (2.8/0.9)</td>
</tr>
<tr>
<td>Average</td>
<td>2.2 (3.1/1.4)</td>
<td>1.6 (2.4/1.5)</td>
</tr>
<tr>
<td>FW1-3</td>
<td>58.3 (7/12)</td>
<td>13.3 (2/15)</td>
</tr>
<tr>
<td>Position 33$^a$</td>
<td>58.3 (7/12)</td>
<td>13.3 (2/15)</td>
</tr>
</tbody>
</table>

Adoptive transfer and primary immunization was performed as described in Supplemental Fig. 1. On day 60 postimmunization, recipient mice were injected with PBS or NP-Ficoll. Five days later, memory B cells (propidium iodide 'CD45.1$^+$CD38$^{\text{high}}$CD138$^-$) were sorted from pooled splenocytes of these mice, from which the B1-8 ki ($V_H$186.2-$\gamma_1$ mRNA sequences were analyzed.

$^a$Average number of mutations per $V_H$ sequence.

$^b$Average numbers of replacement and silent mutations per $V_H$ sequence are in parentheses.

$^c$Percentage of clones carrying tryptophan-to-leucine replacement at codon position 33.
mice with NP-conjugated MSA that forms a TD Ag but is not recognized by mouse T cells because they are tolerant to this self-Ag (Fig. 5A). The NP-MSA challenge did not induce an IgG1 recall response in these mice (data not shown); however, rechallenge of these mice with NP-CGG 3 wk after the secondary challenge induced the same level of anti-NP IgG1 Ab response as in control mice, indicating that NP-MSA did not tolerize memory B cells (Fig. 5B).

To examine the effect of the NP-MSA challenge on Ag-specific IgG1+ memory B cells, we immunized mice containing B1-8ki memory B cells as shown in Fig. 5C and analyzed them in the same way as described in Fig. 4D. Five days after the challenge, the donor-derived IgG1+ memory B cells (CD45.1+CD38highNP+ IgG1+) were present in the mice challenged with NP-MSA at a similar frequency and number to the mock-challenged mice (Fig. 5D, 5E). These data indicate that Ag-induced elimination of Ag-specific memory B cells that we observed with NP-Ficoll challenge is not due to the unavailability of cognate T cell help, but probably depends on the nature of the Ag that is intrinsic to the TI-II Ag (Fig. 6).

Discussion

Unresponsiveness of IgG+ memory B cells to a TI-II Ag

Previous studies have demonstrated that transgenic/knockin B cells expressing IgG1 or chimeric IgM/G1 (containing the transmembrane/cytoplasmic region of IgG1) BCRs exhibit enhanced calcium signaling upon cross-linking in vitro and heightened response to primary TD Ag immunization in vivo (14, 16, 17). In contrast, a recent report demonstrated that B cells expressing a transgenic IgG2a exhibit dampened BCR signaling, leading to poor cell activation and proliferation in vitro and poor response to TI-II Ag immunization in vivo, but nonetheless had an augmented proliferative response to CD40 ligation in vitro and augmented Ab production in TD Ag-immunized mice (33). Together with data showing relatively low expression of these transgenic IgG BCRs, these studies suggest that IgG-BCRs generate spontaneous signals that render naive B cells unresponsive to BCR stimulation alone, similar to B cells rendered anergic by chronic self-Ag stimulation (34), but are hyperresponsive to stimulation by Th cells. However, all of these studies have examined the phenotype of naive B cells expressing the transgenic IgG BCRs, which have survived early developmental perturbation induced by the transgene in the bone marrow (17, 33). Therefore, the above interpretations may not be applicable to normal IgG+ memory B cells. We have clearly shown in this study that TD-Ag (NP-CGG)-induced physiological IgG1+ memory B cells do not clonally expand and develop into plasma cells upon immunization with an epitope-matched TI-II Ag, NP-Ficoll. Although other TI-II Ags would be tested in the future, the data shown in this study indicate that the unresponsiveness of IgG+ B cells to BCR stimulation may stem from the distinctive nature of IgG BCR independent of the cellular context.

Clonal deletion of memory B cells by a TI-II Ag

More importantly, our data demonstrated that the memory B cells expressing an IgG1 BCR with high affinity for the antigenic epitope are eliminated in vivo upon binding with epitope-matched TI-II Ag, resulting in a poor recall response to a subsequent TD Ag rechallenge. This affinity-dependent elimination of memory B cells indicates that the elimination was caused by direct interaction of the BCR with the Ag. The elimination appeared to be due to apoptosis of the memory B cells, and not to anergy, as confirmed by sequence analysis of the remaining repertoire. Memory B cell function was eliminated from splenic B cells purified from primed mice by a brief TI-II Ag treatment in vitro, indicating that the effect is BCR signal-mediated and requires no cells other than B cells. These results together suggest that high-affinity binding to a TI-II Ag by the IgG BCR on memory B cells signals apoptosis, whereas low-affinity binding to the same TI-II Ag is ignored by such cells (Fig. 6). By contrast, both high- and low-affinity binding to TI-I or TD Ags, the latter being provided with cognate T cell help, signal clonal expansion and differentiation. Noticeably, IgG+ memory

FIGURE 5. Tolerance of IgG1+ memory B cells is not induced by a TD Ag that is not recognized by T cells. A and B, C57BL/6 mice were primed with NP-CGG/alum (1) and challenged with NP-conjugated MSA (NP-MSA) or PBS 60 d later (2). Three weeks after the secondary challenge, the mice were challenged again with soluble NP-CGG or PBS (3). Serum anti-NP IgG1 concentrations of the mice (n = 4 per group) were analyzed by ELISA. Data are representative of one of two independent experiments with similar results.

FIGURE 6. Distinct fates of IgG+ memory B cells are determined by the nature of Ags. Newly generated IgG+ memory B cells are either eliminated by apoptosis or left unactivated upon binding with TI-II Ags, depending on the affinity of their BCR for the Ags. In contrast, they respond to form Abs upon binding with TI-I Ags or TD Ags as long as cognate Th help is available. These responses are independent of the affinity of their BCR.
B cells are neither activated nor tolerized upon binding to TD Ags without cognate T cell help. Unlike the memory B cell response, TI-II Ag binding to an IgM BCR on naive B cells, irrespective of its affinity, signals cell activation and proliferation (35) (Fig. 6). Although immature B cells in bone marrow and spleen, but not mature B cells, are known to undergo apoptosis upon BCR cross-linking in vitro, this susceptibility of memory B cells to BCR cross-linking was unknown until this report. To our knowledge, the only other remotely similar previous report demonstrated that human tonsillar IgD memory B cells are susceptible to BCR-induced apoptosis only when they are preactivated through CD40 or BCR in vitro (36).

Mechanism for TI-II–mediated clonal deletion

Previous reports demonstrated that large amounts of multivalent Ag can suppress an ongoing TD immune response and prevent a recall response in mice, although the mechanism for the immunosuppression was not described (37, 38). These studies differ from ours in an important way. These investigators used an extremely high dose (typically 3 mg) of a nonimmunogenic Ag (FITC-conjugated dextran) as a suppressant, whereas we used a rather low dose (25 μg) of TI-II Ag (NP-Ficoll) that is immunogenic in a primary response. Thus, the mechanism for the suppression of memory recall response is likely to be totally different in the two systems. Our study indicated that the Ag binding-induced elimination of memory B cells is not simply due to the lack of concomitant T cell help, because injection of NP-conjugated MSA did not eliminate NP-specific IgG memory B cells. This notion is also supported by the failure to rescue the same memory B cells from NP-Ficoll–induced elimination in vitro by the addition of Th cytokines. Thus, the apoptotic fate of memory B cells is likely determined by the way in which the IgG-BCR binds TI-II Ags, in which each of the highly repetitive epitopes binds to the IgG-BCR with high affinity. How this particular state of binding is translated into an apoptotic signal through IgG, but not IgM, awaits further investigation.

Implications for peripheral self-tolerance

In mice, self-reactive B cells are known to be tolerized at their immature stages either by clonal anergy in the case of soluble self-Ag (39) or by receptor editing in the case in which self-Ags are in a multivalent membrane-bound form (40), and failure to escape self-reactivity by the latter mechanism is resolved by clonal deletion (41, 42). However, B-1 and marginal zone B cells produce IgM Abs that are polyreactive and bind weakly to self-Ag. These natural IgM Abs protect individuals from a variety of pathogens that are polyreactive and bind weakly to self-Ag. These natural IgM Abs could suppress an ongoing TD immune response and prevent a recall response in mice, although the mechanism for the immunosuppression was not described (37, 38). These studies differ from ours in an important way. These investigators used an extremely high dose (typically 3 mg) of a nonimmunogenic Ag (FITC-conjugated dextran) as a suppressant, whereas we used a rather low dose (25 μg) of TI-II Ag (NP-Ficoll) that is immunogenic in a primary response. Thus, the mechanism for the suppression of memory recall response is likely to be totally different in the two systems. Our study indicated that the Ag binding-induced elimination of memory B cells is not simply due to the lack of concomitant T cell help, because injection of NP-conjugated MSA did not eliminate NP-specific IgG memory B cells. This notion is also supported by the failure to rescue the same memory B cells from NP-Ficoll–induced elimination in vitro by the addition of Th cytokines. Thus, the apoptotic fate of memory B cells is likely determined by the way in which the IgG-BCR binds TI-II Ags, in which each of the highly repetitive epitopes binds to the IgG-BCR with high affinity. How this particular state of binding is translated into an apoptotic signal through IgG, but not IgM, awaits further investigation.

Acknowledgments

We thank Takachika Azuma and Akikazu Murakami (Research Institute for Biological Sciences) for reagents and advice on immunization, Klaus Rajewsky (Harvard University) and Mitsuori Maruyama (National Institute for Longevity Sciences) for B-1–8 mice, Hidehiro Kishimoto and Takehiro Hirayama (Research Institute for Biological Sciences) for technical advice, and Peter D. Burrows for critical comments on the manuscript.

Disclosures

The authors have no financial conflicts of interest.

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