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Lyn Signaling To Upregulate GANP Is Critical for the Survival of High-Affinity B Cells in Germinal Centers of Lymphoid Organs

Kazuhiko Kuwahara,*1 Teruo Nakaya,*1 Suchada Phimsen,* Teppei Toda,* Masahiro Kitabatake,* Tomohiro Kaji,† Toshitada Takemori,† Takeshi Watanabe,‡ and Nobuo Sakaguchi*

Signals through BCR and costimulatory molecules play essential roles in selecting high-affinity B cells with Ig V-region mutations in the germinal centers (GCs) of peripheral lymphoid organs. Lyn-deficient (lyn−/−) mice show impaired BCR signal triggering for cell proliferation and GC formation, causing hyper-IgM, and display autoimmunity after aging. In this study, we demonstrate that Lyn-mediated signaling to upregulate GANP is essential for the survival of mature GC-like (mGC) B cells with high-affinity type BCR mutations upon Ag immunization. Transgenic ganp expression into lyn−/− mice did not recover the Lyn-deficient phenotype with regard to B cell differentiation, serum Igs, and impaired GC formation in spleens after immunization with nitrophenyl-chicken γ-globulin, but it markedly rescued cell survival of mGC B cells by suppressing DNA damage, thereby increasing the frequency of the Trp13-to-Leu mutation in the IgVH-186.2 region and affinity maturation of nitrophenyl-binding B cells. GANP may play a critical role in Lyn-mediated signaling for the selection of high-affinity B cells in peripheral lymphoid organs. The Journal of Immunology, 2012, 189: 3472–3479.

A ntilgen stimulation triggers BCR to induce the rapid proliferation of specific B cells for clonal expansion in the germinal center (GC) region with costimulatory signals from Th cells (1, 2). The GC B cells undergo somatic hypermutation (SHM) of IgV regions and class switch recombination (CSR) in S regions by inducing activation-induced cytidine deaminase (AID) (3, 4). The GC B cells with high-affinity BCRs are positively selected through interactions with opsonized Ags captured by the complement receptor 1 on follicular dendritic cells (5, 6). Clonal expansion and maturation of Ag-specific B cells are associated with GC formation, but the IgV-region SHM is generated in mutant mice lacking GC formation (7) or independently from GCs (8, 9) by stimulation with T cell-independent Ags, suggesting that the generation of IgV-region SHM is not directly associated with GC formation in peripheral lymphoid organs. In fact, SHM and CSR can be induced in vitro by AID (10, 11).

Mature B cells in the periphery require the BCR signal for survival. During affinity maturation, the interaction of Ags with the BCR complex is presumably necessary for the selection of high-affinity Abs, which may elicit a critical BCR-signaling cascade. BCR stimulation triggers tyrosine phosphorylation of the ITAM of the B cell-specific molecules as CD79a (12) and CD79b (13), where src-type tyrosine kinases and adaptor molecules accumulate through tyrosine phosphorylation of their SH2 domains (14). The nonreceptor-type tyrosine kinase Lyn is expressed in lymphocytes and plays a role as a proximal tyrosine kinase in BCR-mediated signal transduction, including Syk tyrosine kinase, leading to the activation of Btk, PI3K, and phospholipid Cγ (15). Lyn-deficient (lyn−/−) mice exhibit an immunodeficiency: notably a reduced number of peripheral B cells, dysfunctional BCR signaling, a decreased response to CD40L, and a lack of GC formation, as well as splenomegaly with age and the production of autoantibodies, causing an autoimmune lupus-like phenotype (16, 17).

RNA microarray analysis demonstrated significantly decreased genes in lyn−/− DT40 cells for BCR signaling (CD74, CD22, IgH-C, and CD79b), proliferation (GC-associated nuclear protein [GANP], tumor-associated MAGE like, nucleoside diphosphate kinase, stathmin, prothymosin, and transglutaminase), control of transcription (EF1α, cleavage and polyadenylation specificity factor, CEBP, TCF1, OBF-1, ICSBP, and NF-κB), and cytoskeletal organization (γ-actin, transglutaminase, chaperonin CCTβ, and stathmin) (18). Further analysis revealed that GANP is a highly significant target of Lyn-mediated signaling. GANP is increased in GCs (19) and involved in selective mRNA export (20). B cell-specific ganp-deficient mice (21) demonstrated that

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The online version of this article contains supplemental material.

Abbreviations used in this article: 7AAD, 7-aminoactinomycin D; AID, activation-induced cytidine deaminase; ASC, Ab-secreting cell; CSR, class switch recombination; DSB, dsDNA break; GANP, germinal center-associated nuclear protein; GC, germinal center; MGCR, mature germinal center-like; NIP, 4-hydroxy-5-iodo-3-nitrophenylacetamid; NP, 4-hydroxy-5-iodo-3-nitrophenyl; NP-CG, 4-hydroxy-3-nitrophenyl coupled to chicken γ-globulin; PNA, peanut agglutinin; SHM, somatic hypermutation; TD, T cell dependent.
GANP plays a critical role for generation of high-affinity Abs, a finding that was reciprocally confirmed in B cell-specific gamp-transgenic (gamp<sup>2/2</sup>) mice (22).

In this study, we demonstrate that Lyn-mediated signaling to upregulate GANP plays a crucial role in the survival of mature GC-like (mGC) B cells and the generation of high-affinity B cells in peripheral lymphoid organs.

**Materials and Methods**

**Mice**

Lyn<sup>−/−</sup> and gamp<sup>2x</sup> mice of C57BL/6 background were prepared (17, 22) and maintained in a specific pathogen-free condition in accordance with institutional regulations at the Graduate School of Medical Sciences, Kumamoto University (Kumamoto, Japan).

**Real-time PCR**

PCR was performed using a LightCycler (Roche Molecular Biochemicals, Basel, Switzerland) with oligonucleotide primers and probes obtained from Nihon Gene Research Laboratories (Sendai, Japan). Specific oligonucleotide primers and probes were synthesized as follows: gamp sense, 5'-TGGTTGGGACGACAAAACTG-3'; gamp anti-sense, 5'-CGGCCCAATAAACACACTGAC-3'; gamp donor probe (labeled with fluorescein at its 3' end), 5'-GTCCTCAGTGTAGCCCAAGA-3'; gamp acceptor probe (labeled with LC Red 640 at its 5' end), 5'-GAACTCGTGCGCATTCTTGAAACCGGA-3'; and gamp acceptor probe, 5'-CATCAAGAAAGTGCTGAAGCAGGCATCT-3'. To ensure the fidelity of mRNA extraction and reverse transcription, all samples were subjected to PCR amplification with oligonucleotide primers and probes specific for the constitutively expressed gene gapdh and normalized. The gapdh primers and probes used were as follows: gapdh sense, 5'-GTTCTTACCCCCAATGTTGC-3'; gapdh anti-sense, 5'-GTCCTCAGTGTAGCCCAAGA-3'; gapdh donor probe, 5'-CGCCCTGGGAAACACTGCGATATG-3'; and gapdh acceptor probe, 5'-CATCAAGAAAGTGCTGAAGCAGGCATCT-3'. Gadd45α transcripts were measured using TaqMan gene-expression assays (gadd45α, Hs00169255; and Gadd45α, Hs03929097), the ABI7500 Sequence Detection System, and Sequence Detection System software (all from Applied Biosystems, Foster, CA).

**Cell-proliferation assay**

Spleen B cells (2 × 10<sup>5</sup>/well) were purified using a MACS B cell isolation kit (Miltenyi Biotech, Bergisch Gladbach, Germany) and cultured with F(ab)<sub>2'</sub> fragment of affinity-purified goat anti-mouse IgM (anti-μ) Ab (10 μg/ml). Cells were pulsed with [3H]thymidine deoxyribose (0.2 μCi/well; FCS Ratios Inc., Irvine, CA), and [H] incorporation was measured. For the CFSE-dilution assay, purified B cells were labeled with 1.25 μM CFSE (Invitrogen), followed by anti-μ stimulation for 48 h.

**Activation-induced cell death by BCR cross-linkage**

B cells were stimulated with anti-μ (10 μg/ml) for 24 h, washed with PBS, and incubated at room temperature for 15 min with Annexin V-FITC. Cells were stained with propidium iodide and analyzed by FACSCalibur (BD, Franklin Lakes, NJ).

**Flow cytometric analysis**

Cells washed with PBS containing 2% FCS and 0.05% sodium azide were incubated with mAbs conjugated with FITC, PE, PerCP, and allophycocyanin from eBioscience (San Diego, CA) for B220, Fas, and GL-7. Anti-λ chain mAb (LS136) was from Dr. K. Rajewsky (University of Cologne, Cologne, Germany). PE-conjugated (4-hydroxy-5-iodo-3-nitrophénylacetyl) (NIP) was used (23). 7-Aminoactinomycin D (7AAD) staining was performed after permeabilization.

**Immunohistochemistry**

Frozen sections (6 μm) were fixed with acetone and stained with rat anti-IgD mAb (CS/15) and alkaline phosphatase-conjugated anti-rat IgG Ab. After development using a Vector Blue kit, the sections were stained with biotin-labeled peanut agglutinin (PNA; Vector Laboratories, Burlingame, CA) and HRP-conjugated streptavidin (Kirkegaard & Perry Laboratories, Gaithersburg, MD) and developed by 3,3'-diaminobenzidine tetrahydrochloride. Frozen sections (8 μm) were double immunostained with anti-κ or anti-λ chain-specific mAb (Ac146) with Alexa Fluor 488-conjugated goat anti-rat IgG (Invitrogen, Carlsbad, CA) and biotin-labeled anti-IgD with Alexa Fluor 594-conjugated streptavidin (Invitrogen). The slides were mounted in 2.5% 1,4-diazabicyclo[2.2.2]octan/80% glycerol and observed using a fluorescence microscope (FV500; Olympus, Tokyo, Japan).

**Immunization of mice**

Mice (8–12 wk old) were immunized with T cell-dependent (TD) Ags, 4-hydroxy-3-nitrophenyl (NP) coupled to chicken γ-globulin (NP:CG; Biosearch Technologies, Novato, CA) (100 μg) precipitated with alum, or SRBCs (0.3 ml 2% solution in PBS).

**Analyses of mGC B cells**

The age- and sex-matched lyn<sup>−/−</sup>, lyn<sup>−/−</sup>, and gamp<sup>2/2</sup>lyn<sup>−/−</sup> mice were immunized with TD Ags, SRBCs, or NP-CG. Spleens were obtained at day 10, and B220<sup>+</sup> B cells were stained with allophycocyanin-conjugated anti-B220, PE-conjugated anti-Fas, and FITC-conjugated anti-GL-7 to enrich the B220<sup>+</sup>Fas<sup>+</sup>GL-7<sup>+</sup> cells as mGC B cells using a JSAN cell sorter (BayBioscience, Kobe, Japan). The mGC B cells were yielded as small B cells after immunization with TD Ags (0.8–2.5% against NP-CG and 3–5% against SRBCs) (Fig. 2B, 2D), and cell proportion (%), NIP-binding assays, viability, and apoptosis were analyzed by 7AAD staining of B220<sup>+</sup>GL-7<sup>+</sup> cells cultured with anti-CD40 (10 μg/ml) for 6 h without anti-Fas.

**BrdU labeling**

The lyn<sup>−/−</sup>, lyn<sup>−/−</sup>, and gamp<sup>2/2</sup>lyn<sup>−/−</sup> (10-wk-old) mice were immunized with SRBCs for 10 d. BrdU (1 mg/mouse) was injected 24 h before flow cytometric analysis. Cells were stained by anti-BrdU mAb (BD), together with B220<sup>+</sup>Fas<sup>+</sup>GL-7<sup>+</sup> markers, and analyzed using a FACSCalibur with FlowJo software.

**Sequencing of V<sub>G</sub>-186.2 mRNA**

V<sub>G</sub>-186.2 transcripts were amplified as described (22) from cDNAs synthesized using oligo-dT–primed SuperScript III (Invitrogen) with total RNAs and cloned for sequencing.

**Alkaline Comet assay**

B cells were subjected to Comet assay, according to the manufacturer’s instructions (Trevigen, Gaithersburg, MD). Cells were mixed with molten LM Agarose, transferred onto Comet Slides, and immersed in prechilled Lysis Solution and then in alkaline solution (pH > 13) at room temperature in the dark. Slides were electrophoresed with an alkaline solution (300 mM NaOH, 1 mM EDTA [pH > 13]), immersed in distilled water, 70% ethanol, and stained with SYBR Green I at 4˚C for 5 min. Damaged cells were measured using a free software program (CometScore) by three independent assays for statistical evaluation.

**ELISPOT assay**

Ninety-six-well MultiScreen Filter Plates (Millipore, Billerica, MA) were coated with 50 μg/ml NP2-BSA or NP<sub>2</sub>-BSA for 2 h at room temperature, and the wells were blocked with 1% BSA/PBS for 4 h. Spleen or bone marrow cells (2 × 10<sup>5</sup>) from mice immunized with NP-CG for 42 d were added to the NP-BSA–coated wells and incubated for 3 h at 37˚C, 5% CO<sub>2</sub>. The plates were washed, blocked with 1% BSA/PBS, and incubated for 2 h with alkaline phosphatase-conjugated anti-IgG<sub>1</sub> Ab (Southern Biotech, Birmingham, AL). Bound secondary Ab was assessed by incubation with Vector blue substrate. After development, blue spots were counted.

**Statistical analysis**

Statistical analyses were performed using the SPSS statistical program package (SPSS, Chicago, IL). Distortion values among the groups were analyzed using one-way repeated-measures ANOVA at a 0.05 level of significance. The Tukey post hoc test was performed to identify multiple comparisons. The results were considered significant when p < 0.05.

**Results**

Transgenic gamp expression partially recovers the Lyn-deficient phenotype in lyn<sup>−/−</sup> mice

The lyn<sup>−/−</sup> mice (17) were crossed with transgenic mice expressing gamp transcripts in B lineage cells (22) to establish gamp<sup>2/2</sup>lyn<sup>−/−</sup> mice. Spleens from gamp<sup>2/2</sup>lyn<sup>−/−</sup> mice were similar in size to those of control heterozygous Lyn-deficient (lyn<sup>+/−</sup>) mice compared with the enlarged spleens of lyn<sup>−/−</sup> mice (Fig. 1A) (17). B cells of lyn<sup>−/−</sup>...
mice express ganp transcripts at a low level (20%) compared with control lyn−/− mice (p < 0.002). The amount of ganp transcripts in B cells in ganpTg lyn−/− mice was increased to 65% of that in control lyn−/− mice, with a significant increase compared with that of lyn−/− mice (p < 0.002, Fig. 1B). Although the spleen size was enlarged in lyn−/− mice, the total number of spleen cells was decreased, with a proportional decrease in the number of spleen B cells compared with those in control lyn−/− mice, as reported previously (17). Transgenic ganp expression did not significantly change the number of spleen B cells observed in lyn−/− mice (Supplemental Table 1).

We compared the response of B cells from these mice with anti-μ Ab stimulation in vitro. Similar to lyn−/− mice, ganpTg lyn−/− mice did not show B cell proliferation at 48 h after stimulation with anti-μ Ab (Fig. 1C), but they showed significant B cell proliferation with LPS, LPS + IL-4, anti-CD40, and anti-μ + anti-CD40 compared with lyn−/− mice (Supplemental Fig. 1A). No difference was observed in the induction of activation-induced cell death after 24 h by BCR cross-linkage of B cells from lyn−/− and ganpTg lyn−/− mice (Fig. 1D), and Fas upregulation also was not different after stimulation with anti-CD40 + anti-μ, anti-CD40 + IL-4, and anti-CD40 + anti-μ + IL-4 on B cells from lyn−/− and ganpTg lyn−/− mice compared with those from control lyn−/− mice (Supplemental Fig. 1B). Cell division of anti-μ-stimulated B cells was evaluated using a CFSE-dilution assay. Spleen B cells from lyn−/− and ganpTg lyn−/− mice showed significantly impaired cell division at 48 h (Fig. 1E). Supporting this result, the number of live cells stimulated with anti-μ Ab in lyn−/− and ganpTg lyn−/− mice was decreased compared with those in lyn−/− mice at 48 h (Fig. 1F).

The lyn−/− mice displayed high levels of serum IgM and anti-dsDNA Abs of both IgM and IgG (16, 17). GanpTg lyn−/− mice did not exhibit a reduction in serum IgM level or anti-dsDNA Ab of IgM or IgG compared with lyn−/− mice (data not shown). In addition, introduction of the ganp transgene did not recover glomerulonephritis with hyperlobular and hypercellular glomeruli in the kidneys of aged lyn−/− mice (data not shown). The ganp-transgenic expression partially recovers the Lyn-deficient phenotype in B cell proliferation in vitro, but it does not rescue the hyper-IgM phenotype associated with the production of autoantibodies in mice.

GanpTg lyn−/− mice do not develop GCs but rescue the survival of mGC B cells

Ten days after immunization with SRBCs, the control lyn−/− mice created mature GCs with PNA staining in the spleen, but neither lyn−/− nor ganpTg lyn−/− mice showed similarly mature PNA+ areas surrounded by IgD+ follicular B cells (Fig. 2A). The mGC B cells enriched with B220+Fas+GL-7+ marker (Fig. 2B) showed similar levels of ganp transcripts compared with that of total spleen B cells (Fig. 2C). The number of mGC B cells was decreased in the spleens of lyn−/− mice (by 1–4%) compared with that in lyn+/+ mice after immunization with SRBCs (Fig. 2D). Such mGC B cells increased modestly in ganpTg lyn−/− mice, although the difference was not statistically significant. mGC B cells from lyn−/− or ganpTg lyn−/− mice showed decreased BrdU incorporation compared with control mice (Fig. 2E). These results indicate that Lyn deficiency disturbed the generation of mGC B cells, leading to impaired development of mature GCs upon immunization with TD Ag.

**FIGURE 1.** Effect of ganp introduction into lyn−/− mice. (A) Spleens from age- and sex-matched mice (left panel). Average lengths of spleens (right panel). (B) The ganp transcripts were measured in B cells from lyn−/−, lyn−/−, and ganpTg lyn−/− mice. Data are results of three samples/experiment that were repeated four times. (C) B cells from lyn−/− mice, lyn−/− mice, and ganpTg lyn−/− mice were stimulated with anti-μ in comparison with medium alone (N.S.), and [3H]Tdr incorporation was measured. All assays were performed in triplicate and repeated four times. (D) Anti-μM-induced activation-induced cell death in B cells. B cells were stimulated with anti-μ for 24 h. The surviving cells were defined as the Annexin V+ propidium iodide− population. (E) Cell division after anti-μ stimulation was evaluated using a CFSE-dilution assay. CFSE intensity without cell proliferation is represented by the shaded graph. (F) Number of B cells after anti-μ stimulation. At 24 and 48 h after stimulation, live cells were counted by dye-exclusion assay. All assays were performed in triplicate and repeated three times. **p < 0.02, ***p < 0.002.
Transgenic ganp expression maintains mGC B cells by suppressing DNA damage in mice

mGC B cells were cultured with anti-CD40 in vitro to compare the survival by 7AAD staining. The mGC B cells were highly sensitive to apoptosis, displaying >84% apoptotic cells during the 6-h culture without anti-CD40 in control lyn−/− mice (data not shown). This effect was more severe in mGC B cells of lyn−/− mice, but the mGC B cells from ganp+ lyn−/− mice showed fewer apoptotic cells and more survived, which became more evident by gating the activated B cell population stimulated with anti-CD40. Survival (7AAD− cells) of activated mGC B cells in ganp+ lyn−/− mice recovered from 60 to 94%, similar to the level in control lyn−/− mice (87%; Fig. 2F, white bar). GANP upregulation maintained the survival of mGC B cells, which are extremely sensitive to apoptosis in lyn−/− mice.

The frequencies of live cells among the mGC B cell population in mutant mice were compared with that in control lyn−/− mice (Fig. 3A), a finding that was further supported by the decrease in active caspase 3+ cells among mGC B cells in ganp+ lyn−/− mice (Fig. 3B). This change also reflected the upregulation of gadd45a transcription that was presumably caused by p53 as a consequence of apoptotic DNA damage (Fig. 3C).

We examined whether GANP plays a role in regulation of DNA damages in mGC B cells. mGC B cells were obtained from mutants and control mice after immunization with SRBCs, and the DNA damage was measured directly using Comet assay (Fig. 3D). DNA damage was detected in most mGC B cells from lyn−/− mice (93%); this frequency was greater than that in control lyn−/− mice (45%) (p < 0.002). Transgenic ganp expression significantly suppressed DNA damage in ganp+ lyn−/− mice (from 93 to 64%) (p < 0.002). The lyn−/− mice showed a high tail moment value (18 U) compared with control lyn−/− (7.8 U) and ganp+ lyn−/− (9 U) mice (both p < 0.002). Lyn-mediated signaling to upregulate GANP is critical to prevent widespread DNA damage in mGC B cells during immune responses.
were localized outside of the IgD+ follicular region in the mutant
fore, we sorted the NIP-binding (NIP+) mGC B cell population of
** from three independent experiments are shown.
metric analysis, as reported previously (24). The
B cells showed similar mutation frequencies in the
region was sequenced (Table I). The mutant spleen mGC
mice were immunized with SRBCs for 10 d, and the
mice were stimulated with anti-CD40 and analyzed by
Comet assay with SYBR Green (Fig. 4A). The proportion of
 lyn2 mice recovered significantly
(p < 0.02), comparable to that in control lyn+/− mice (Fig. 4B),
although the total number of mGC B cells decreased similarly
compared with lyn−/− mice (Supplemental Table I). Immunohis-
tochemical analysis revealed cells stained with anti-λ-chain or
Ac146 against an IgVH-186.2 idiotype (Fig. 4C). The Igλ+ cells were
localized outside of the IgD+ follicular region in the mutant
mice (indicated by arrowheads in the magnified photomicrographs),
in marked contrast to control lyn+/− mice (Fig. 4C, middle panel).
Ac146+ cells were detected outside of the IgD+ follicular region in
lyn−/− and ganp+lyn− mice (Fig. 4C, bottom panel).

Given the recovery of NP-binding mGC B cell frequency in
ganp+lyn− mice, we compared the mutation frequencies and the
profiles of the IgVH186.2 region in mGC B cells. First, mGC B
cells were sorted by the B220+ Fas+GL7+ marker, and the IgVH-
186.2 region was sequenced (Table I). The mutant spleen mGC
B cells showed similar mutation frequencies in the
IgVH186.2 region sequences compared with those of control mice, confirming
that the SHM-inducing potential is not impaired by the Lyn de-
iciency (Supplemental Table II), as reported previously (24). The
IgVH186.2 region sequences are derived from two kinds of mGC
B cell populations: B cells reactive to NP-hapten, as well as the
B cells that have altered the Ag specificity by the SHM. Impor-
tantly, the decrease in the unique mutation at Trp33 to Leu, im-
plying affinity maturation, was significantly recovered in Lyn-
deficient B cells by the ganp-transgene (from 7 to 28%). There-
fore, we sorted the NIP-binding (NIP+) mGC B cell population of
the mutant and control mice (Table II). The overall average mu-
tation rate was decreased in the NIP+ population of lyn−/− mice
(1.9 mutations/IgVH186.2 sequence), but it was recovered in
ganp+lyn− mice (3.2 mutations) to a level comparable to that in
control mice (3.7 mutations). NIP+ mGC B cells from ganp+lyn−/− mice showed a significant increase in the replacement (R) to silence
(S) mutation rate (R/S ratio) (10.0:1) at CDR1 and CDR2; more-
over, it increased the rate of the Trp33 to Leu mutation (23%) to
a level greater than that in lyn−/− mice (Table II). The lyn−/− mice showed virtually no Trp33 to Leu mutation in the NIP+ mGC B cells.
The mutation profile indicated that the ganp+lyn−/− mice could enrich the high-affinity-type Trp33 to Leu mutation in the NIP+
mGC B cell population during the maturation in GCs (Supplemental
Fig. 2). Taken together, the results indicated that Lyn-mediated
signaling to upregulate GANP, which could be mimicked by the
 ganp transgene, serves to generate high-affinity Ag-binding B cells,
even in the extrafollicular region, in peripheral lymphoid organs.

Furthermore, we examined the effect of GANP on the differ-
etiation of NP-binding B cells into Ab-secreting cells (ASCs).
Upon immunization with soluble Ag, naive Ag-reactive B cells proliferated rapidly in the dark zone and then differentiated in the
light zone of the GCs undergoing the maturation process to
express high-affinity and class-switched BCRs. The GC reaction presumably occurs through multiple-round processes during the
immune response, remaining as mGC B cells to gain better affinity
or alternatively differentiating into long-lived plasma cells in the
spleen that later appear in the bone marrow (25). The ASCs re-
directive to the Ag with a low amount of NP are estimated to produce
high-affinity anti-NP Ab of the IgG1 isotype. The low-affinity
ASCs measured by the NP17-coated plate were comparable be-
tween control and mutant mice, but the high-affinity ASCs mea-
sured by the NP2-coated plate were clearly decreased in the
spleens of lyn−/− mice and were decreased to a lesser extent in the
bone marrow (Fig. 5, left and middle panels). The corrected per-
centages, based on the ratio of high- and low-affinity ASCs in each
assay, clearly demonstrated that Lyn deficiency impaired the
generation of high-affinity Ab-producing cells, which was indeed
recovered by the transgenic expression of GANP (Fig. 5, right
panel).

Transgenic ganp expression maintains mGC B cells expressing
Ag-specific BCR
We further investigated whether Ag-reactive B cells were indeed
maintained in ganp+lyn− mice. Interestingly, in a flow cyto-
metric analysis, ganp+lyn− mice recovered the Igα-chain usage
to a similar level as did lyn−/− mice (Fig. 4A). The proportion of
NIP+ B cells from ganp+lyn− mice recovered significantly
(p < 0.02), comparable to that in control lyn+/− mice (Fig. 4B),
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Transgenic introduction of the ganp gene into lyn-/- mice mimicked the Ag-driven upregulation of GANP in mGC B cells in vivo and partially recovered the Lyn-deficient phenotype. Transgenic GANP expression did not recover the hyper-IgM of Lyn deficiency, indicating that the elimination of autoantibody-producing B cells might be dependent on other Lyn-mediated signaling associated with the formation of mature GCs. However, experiments in lyn-/- and ganp^Tg lyn-/- mice revealed several important findings regarding the generation of high-affinity B cells in GCs. The generation of SHM at the IgVH region was not affected by Lyn deficiency, as reported previously (24). B cells with high-

Table I. SHM at the VH-186.2 region of mGC B cells after immunization with NP-CG

<table>
<thead>
<tr>
<th>Parameters</th>
<th>lyn+/-</th>
<th>lyn-/-</th>
<th>ganp^Tg lyn-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of V_H-186.2 sequences examined</td>
<td>44</td>
<td>41</td>
<td>40</td>
</tr>
<tr>
<td>Average mutation (range)^a</td>
<td>3.4 (1–15)</td>
<td>(0–14)</td>
<td>(0–7)</td>
</tr>
<tr>
<td>Transition/transversion ratio</td>
<td>1.01</td>
<td>1.71</td>
<td>0.97</td>
</tr>
<tr>
<td>R/S ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDR1 and 2</td>
<td>10.1:1</td>
<td>5.9:1</td>
<td>5.1:1</td>
</tr>
<tr>
<td>FR1–3</td>
<td>3.1:1</td>
<td>2.0:1</td>
<td>1.6:1</td>
</tr>
<tr>
<td>Mutation (Trp → Leu) at position 33 (%)</td>
<td>55</td>
<td>7</td>
<td>28</td>
</tr>
</tbody>
</table>

The control and mutant mice (12–14 wk old) were immunized with NP-CG for 10 d. The B220^+ Fas^+ GL-7^+ mGC B cells of the spleens were sorted by flow cytometry. Total RNAs were purified and subjected to cloning and sequencing for VH-186.2 region. Five mice/genotype were used.

The mutation frequencies were determined based on the genomic VH-186.2 sequence of C57BL/6 mice.

The mutations were highly concentrated in the CDR or framework region (FR), with a clustering of R mutations in the CDR but only a few in the FR. Therefore, the R to S mutation rate (R/S ratio) is generally higher in the CDR than in the FR.

Table II. SHM at the VH-186.2 region in the NIP- mGC B cell population

<table>
<thead>
<tr>
<th>Parameters</th>
<th>lyn+/-</th>
<th>lyn-/-</th>
<th>ganp^Tg lyn-/-</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of V_H-186.2 sequences examined</td>
<td>43</td>
<td>42</td>
<td>47</td>
</tr>
<tr>
<td>Average mutation (range)^a</td>
<td>3.7 (1–9)</td>
<td>(0–5)</td>
<td>(0–8)</td>
</tr>
<tr>
<td>Transition/transversion ratio</td>
<td>0.7</td>
<td>2.1</td>
<td>1.5</td>
</tr>
<tr>
<td>R/S ratio</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CDR1 and 2</td>
<td>10.3:1</td>
<td>15.0:1</td>
<td>10.0:1</td>
</tr>
<tr>
<td>FR1–3</td>
<td>2.9:1</td>
<td>9.0:1</td>
<td>2.4:1</td>
</tr>
<tr>
<td>Mutation (Trp → Leu) at position 33 (%)</td>
<td>75</td>
<td>0</td>
<td>23</td>
</tr>
</tbody>
</table>

The control and mutant mice (12–14 wk old) were immunized with NP-CG in alum for 10 d. Five mice/genotype were used.

The mutation frequencies were determined based on the genomic VH-186.2 sequence of C57BL/6 mice.

The R/S ratio was calculated using the same method described in Table I.

FR, Framework region.
affinity-type BCRs were decreased in the GCs of Lyn-deficient mice. The loss of the high-affinity-type mutation in mGC B cells of lyn<sup>−/−</sup> mice was attributed to their impaired proliferation and survival in the lyn<sup>−/−</sup> mouse spleen. Particularly, the mGC B cells became sensitive to apoptosis as a result of the absence of Lyn-mediated signaling, irrespective of the presence or absence of the GC architecture.

BCR-mediated signal transduction induces B cell proliferation and is involved in the generation of the IgV<sub>H</sub>-region SHM and CSR at the S regions in GCs (3). Lyn deficiency impaired GC formation, but it induced SHM of the IgV<sub>H</sub>-region at a frequency similar to that in control mice, suggesting that non-Lyn–associated BCR-mediated signal transduction induces AID to generate the IgV<sub>H</sub>-region SHM. The lack of Lyn led to a significant decrease in mGC B cells expressing the B220<sup>+</sup>Fas<sup>+</sup>GL-7<sup>+</sup> phenotype in the spleen after immunization with TD Ag (Fig. 2D, Supplemental Table I). However, B cells with the high-affinity–type mutation at the IgV<sub>H</sub>-region were markedly decreased in the mGC B cell population (Tables I, II), suggesting that the BCR-associated Lyn-mediated signal is essential for maintaining high-affinity B cells as the positive selection.

BCR-mediated Lyn signaling leads to the upregulation of PU.1, which is the transcription factor for ganp gene expression in B cells (18, 26). PU.1 and its close relative Spi-B are members of the Ets domain-containing transcription factor family that is expressed in hematopoietic cells, including B cells, T cells, and macrophages (27). The specific deletion of PU.1 in the B lineage did not markedly affect B cell development (28). However, although the investigators did not pay much attention in the details of B cell populations, their results revealed that follicular B cells were markedly reduced (to half) in mutant mice (17). The specific deletion of PU.1 in the B lineage did not markedly affect B cell development (28).

mGC B cells from lyn<sup>−/−</sup> or ganp<sup>Tg</sup>lyn<sup>−/−</sup> mice were generated in a similar proportion to spleen B cells compared with control mice upon immunization with TD Ag, but they showed a marked decrease in BrdU incorporation, indicating impaired cell proliferation (Fig. 2E). The Ag-driven B cells were dispersed in the extrafollicular region of lyn<sup>−/−</sup> mice, indicating that the generation of SHM at the IgV<sub>H</sub>-region occurs in B cells independently from Lyn-mediated signaling, but further activation or cell maintenance requires Lyn-mediated signaling and, presumably, GC formation. In addition, GANP upregulation is not sufficient for the negative selection of B cells in GCs, indicating that Lyn-mediated signaling to other target molecules might be necessary to eliminate autoreactive B cell clones in GCs in vivo.

GANP interacts with AID in the cytoplasm and is involved in recruiting AID to the nucleus and to the IgV<sub>H</sub>-region DNA in B cells (29). GANP is involved in mRNA export in mammalian cells (30) and particularly in the selective export of Slugosinh-1 mRNA required for cell cycle progression through the M phase (20). The lack of GANP results in embryonic lethality at embryonic day 12.5 in mice and induces homology-mediated DNA hyperrecombination in cells, similar to that observed in suppressor of actin 3-deficient yeast cells (31, 32). Alterations in GANP expression regulate the dsDNA breaks (DSB) at the IgV<sub>H</sub>-region in GC B cells (33), suggesting involvement of GANP in the generation of DSBs or in DNA repair after translocation of the GANP/AID complex to the target DNA in B cells. The Ag-driven B cells undergo AID-induced IgV-region SHM and S-region CSR, which inevitably accompany DNA damage at a high level during proliferation and differentiation in GCs. Recent studies demonstrated the association of the RNA-splicing mechanism with DSBR (34, 35). GANP may be indirectly or directly involved in the ribonucleoprotein complex associated with transcription-coupled DNA damage. In support of this notion, the level of DNA damage was increased in mGC B cells of lyn<sup>−/−</sup> mice but was significantly suppressed in those of ganp<sup>Tg</sup>lyn<sup>−/−</sup> mice (Fig. 3D). GANP upregulation reduces the amount of DNA damage occurring during IgV-region transcription to a level that can be repaired during the maturation and proliferation of mGC B cells, which is essential for the survival of Ag-specific mGC B cells in the lymphoid organs.

Disclosures

The authors have no financial conflicts of interest.

References