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GANP suppresses the arginine methyltransferase PRMT5 regulating IL-4-mediated STAT6-signaling to IgE production in B cells

Hideya Igarashi^{a,b,1}, Kazuhiko Kuwahara^{a,b}, Mikoto Yoshida^{a,b}, Yan Xing^{a,b},
Kazuhiko Maeda^{a,b}, Koichi Nakajima^c, Nobuo Sakaguchi^{a,b,*}

^a Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan

^b Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Japan

^c Department of Immunology, Osaka City University Graduate School of Medicine, 1-4-3, Asahimachi, Abenoku, Osaka 545-8585, Japan

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ABSTRACT

Antigen (Ag)-driven B cells undergo antibody (Ab) affinity maturation and class switching in germinal center (GC) B cells. GANP is one of the molecules required for Ab affinity maturation. We herein found an increase of IgE in B cell *ganp*-deficient mice and studied the signal transduction pathway regulated by GANP. GANP suppresses the STAT-mediated transcription activity in GC B cells with the regulation of arginine methyltransferase activity by the interaction with JAK-binding protein arginine methyltransferase (PRMT) 5 and JAK1/JAK3 that are responsible for STAT6 activation. The *prmt5* mRNA was up-regulated in B cells after stimulation *in vitro* and *in vivo* in GC B cells. The loss of GANP caused up-regulation of phosphorylation and arginine dimethylation of STAT6 in B cells after stimulation with LPS and IL-4 *in vitro*. On the contrary, GANP over-expressed B cells in *ganp* gene-transgenic mice showed a low STAT6 phosphorylation after stimulation. The over-expression of PRMT5 caused the up-regulation of STAT6-mediated gene transcription, which was also suppressed by the co-transfection of GANP, in luciferase reporter assay. GANP down-regulates JAK1/JAK3 to STAT6-signaling with regulation of arginine methylation activity, which might be responsible for the B cell endogenous suppressive mechanism of hyper-IgE.

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1. Introduction

Antigenic stimulation triggers the proliferation and differentiation of naïve B cells expressing Ag-specific B cell antigen receptor (BCR), with the co-stimulatory signals provided by Th2 and follicular dendritic cells in the GCs of lymphoid follicles, to produce high-affinity and class-switched Abs against T cell dependent (TD) Ags (MacLennan, 1994; Rajewsky, 1996). The Ag-driven B cells undergo *Ig V-region* gene somatic hypermutation (SHM) for affinity maturation and class switch recombination (CSR) from C μ to down-stream C γ -region genes by the inducible expression of activation-induced cytidine deaminase (AID) in GC B cells. The stimulation of B cells with various stimulatory reagents, together with IL-4, induces the germ line transcription of the down-stream C γ region locus and expression of AID that generates DNA damage, thus

resulting in DNA double-strand breaks (Honjo et al., 2002; Dudley et al., 2005). Once matured, the high-affinity, class-switched B cells need to maintain BCR specificity, presumably by suppressing or down-regulating the signals that cause further DNA diversification in GC B cells; however, the molecular mechanism involved in the regulation of signal transduction in the later stage of B cell maturation in GCs remains undetermined.

The JAK/STAT signals are presumably regulated by various mechanisms: namely, phosphorylation, association with other adaptor molecules, protein degradation, and other protein modifications. Among a number of negative regulators of the JAK/STAT-signaling pathways, silencers of cytokine signaling (SOCS) forms a negative feedback loop, in which cytokine stimulation induces SOCS to inhibit the cytokine-mediated signaling pathway (Larsen and Ropke, 2002). Another group of negative regulators are the protein inhibitors of activated STAT (PIAS) protein that bind to phosphorylated STAT dimers, and prevent their binding to DNA transcriptional elements (Shuai and Liu, 2005). STAT1 activity is regulated by methylation at arginine (Arg)-31, which reduces its ability to associate with PIAS. This increases the binding to the DNA element (Rogers et al., 2003), which indicates that arginine methylation plays an important role in the regulation of JAK/STAT-signaling. The Arg-31 is conserved in the STATs, thus suggesting a critical role

* Corresponding author at: Department of Immunology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1, Honjo, Kumamoto 860-8556, Japan.
Tel.: +81 96 373-5135; fax: +81 96 373 5138.

E-mail address: nobusaka@gpo.kumamoto-u.ac.jp (N. Sakaguchi).

¹ Present address: Department of Immunology and Molecular Genetics, Kawasaki Medical School, 577 Matsushima, Kurashiki-city, Okayama 701-0192, Japan.

for arginine methylation in STAT activation, although this subject remains controversial (Komyod et al., 2005). Arg-27 is methylated in STAT6, and has been suggested to play a role in the activation of STAT6 by experiments using mutated STAT6 (Chen et al., 2004). No PIAS has been identified for STAT6, thus protein modification with arginine methylation might play a role via a different mechanism in JAK1 and JAK3/STAT6-signaling pathways.

The differentiation of Ag-driven B cells depends on the expression of several molecules in GCs, including those involved in affinity maturation, induction, and repair of DNA damage, cell-cycle regulation, transcriptional regulation, cell survival, selection for high-affinity B cells, and receptors for chemokines and cytokines (Klein et al., 2003; McHeyzer-Williams, 2003; Wolniak et al., 2004). GANP (GC-associated nuclear protein) is a molecule up-regulated in GC B cells upon immunization with TD Ags, and plays a critical role in the affinity maturation of B cells (Kuwahara et al., 2000, 2004; Sakaguchi et al., 2002, 2005). We herein provide evidence that GANP is physically and functionally associated with arginine methyltransferase PRMT5, and it forms a complex with JAK1 and JAK3, thus suggesting that GANP regulates the JAK/STAT signal transduction pathway in GC B cells through the regulation of arginine methyltransferase PRMT5 activity.

2. Materials and methods

2.1. Preparation of the stable transfectants expressing recombinant GANP protein

Murine *ganp* cDNA was inserted into the cloning site downstream of sequential tags, Flag and calmodulin-binding site, in pEF-BOS-derived mammalian expression vector. The final construct was co-transfected, with the plasmid containing hygromycin resistance gene (pTK-Hyg; Clontech Laboratories, Inc., Mountain View, CA) at a ratio of 10:1, into 293T human embryo kidney cells by a liposome-based gene delivery method (Fugene 6; Roche, Mannheim, Germany), cultured for 48 h in the absence of any drugs; then the culture medium was replaced with one containing hygromycin (Sigma–Aldrich, St Louis, MO) at a concentration of 200 µg/ml, and maintained until the cells grew under this drug selection pressure. The expression of the recombinant GANP protein in the established stable transfectant was evaluated by a Western blot analysis with anti-Flag Ab (Invitrogen, Carlsbad, CA).

2.2. Tandem affinity purification (TAP)

Approximately 1×10^9 of stably transfected 293T cells were solubilized for 1 h in solubilization buffer with 1% TNE lysis buffer (1% Nonidet-P40, 150 mM NaCl, 10 mM Tris–HCl, pH 7.5, 1 mM EDTA), in the presence of a protease inhibitor cocktail (Sigma–Aldrich), at 2×10^7 cells/ml on ice. The crude lysate was kept once at -80°C to raise the solubility. The thawed supernatant recovered after centrifugation at $20,000 \times g$ for 30 min was incubated overnight with 200 µl anti-Flag M2 affinity gel (Sigma–Aldrich). The resin was washed once with 1 ml IPP150 buffer (0.1% Nonidet-P40, 150 mM NaCl, 10 mM Tris–HCl, pH 8.0) and twice with TEV cleavage buffer (0.1% Nonidet-P40, 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 0.5 mM EDTA, 1 mM DTT), then resuspended in 100 µl of the same buffer, and incubated for 2 h at 16°C with 10 U TEV protease (Invitrogen). The supernatant was collected by brief centrifugation, mixed with 900 µl IPP150 calmodulin-binding buffer (0.1% Nonidet-P40, 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM Mg-acetate, 10 mM 2-ME, 1 mM imidazole, and 2 mM CaCl_2), and incubated for 2 h at 4°C with 50 µl calmodulin beads (Stratagene, La Jolla, CA). The beads were washed four times with 1 ml calmodulin-binding buffer, and

the retained proteins were eluted with 250 µl IPP150 calmodulin elution buffer (0.1% Nonidet-P40, 150 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM Mg-acetate, 10 mM 2-ME, 1 mM imidazole, 2 mM EGTA). The eluate was concentrated by protein precipitation with trichloroacetic acid.

2.3. Mass spectrometry and protein identification

The eluted proteins were resolved by SDS-PAGE and visualized with Simply Blue dye (Invitrogen). The stained bands were excised and subjected to in-gel tryptic digestion using modified porcine trypsin (Promega, Madison, WI). The tryptic digest was analyzed by a nanospray Qq-TOF mass spectrometer (QSTAR Pulsar XL; Applied Biosystems, Foster City, CA). The MS/MS data were searched against vertebrate sequences using the Mascot search engine (version 2.1.04).

2.4. Mice

The conditional B-GANP^{−/−} and *ganp* cDNA transgenic mice (GANP^{tg}) were described previously (Fujimura et al., 2003; Kuwahara et al., 2004; Sakaguchi et al., 2005). C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, MA). All mice were maintained in the Center for Animal Resources and Development (CARD), Kumamoto University. All studies and procedures were approved by the Kumamoto University Animal Care and Use Committee.

2.5. Purification of B cell

Eight to 12-week-old mice were sacrificed, and their lymphoid cells were obtained from the spleen. Red blood cells were depleted with ACK (0.15 M NH_4Cl , 10 mM KHCO_3 , 0.1 mM EDTA, pH 7.2) hypotonic lysis buffer. Splenic B cells were isolated by the depletion of CD43⁺, CD4⁺, and Ter-119⁺ cells with magnetic beads (MACS; Miltenyi Biotec, Auburn, CA) and used for all experiments. The isolated B cells were at least 90% B220⁺ as verified by flow cytometry.

2.6. Cell lines and cell culture

The purified splenic B cells and IgG⁺ mature B cell line M12 were maintained in RPMI-1640 medium (Sigma–Aldrich) supplemented with 10% heat-inactivated FCS, 2-ME (5×10^{-7} M), streptomycin (100 µg/ml), penicillin (100 U/ml), and 2 mM L-glutamine in the 5% CO_2 -humidified incubator at 37°C . HepG2, 293T, and COS7 cells were maintained in Dulbecco's MEM (Sigma–Aldrich) with the same supplements in the 10% CO_2 -humidified incubator at 37°C . To stimulate cells, 1×10^6 cells of splenic B cells or M12 were stimulated with LPS (10 µg/ml, from *Escherichia coli* 055:B5, Sigma–Aldrich), recombinant murine IL-4 (mIL-4, 100 U/ml, R&D, Minneapolis, MN), recombinant human IL-4 (hIL-4, 10 ng/ml, Pepro Tech, Rocky Hill, NJ), goat anti-mouse IgM (Fab')₂ Ab (10 µg/ml, ICN Biomedicals, Inc., Costa Mesa, CA), and/or rat anti-mouse CD40 Ab (5 µg/ml, clone: LB429) (Nomura et al., 1995).

2.7. Determination of Ig titers

The Ig titers of the sera were measured using Mouse Ig Quantitation Kit for IgG₃, IgG₁, IgE, and IgA (Bethyl, Montgomery, TX) by a spectrophotometer (Nalge Nunc International, Rochester, NY). The Ig concentration was quantified against the standard samples of the kits.

2.8. Immunization and cell sorting

The mice were immunized peritoneally with TD-Ag, sheep red blood cell (SRBC; Nippon Bio-Test Laboratories, Tokyo, Japan), or NP₃₆-CG (Biosearch Technologies, Novato, CA) precipitated with alum, for 9 d as described (Kuwahara et al., 2004). The purified splenic B cells from the immunized mice were stained with FITC-labeled Ab against mouse T cell- and B cell-activation antigen (GL7; commonly used as a marker for mouse germinal center B cells) and APC-anti-mouse B220 Ab (eBioscience, Inc., San Diego, CA). GL7⁺B220⁺ GC B cells and GL7[−]B220⁺ non-GC B cells were isolated on a JSAN cell sorter system (BayBio Science, Kobe, Japan).

2.9. Immunoprecipitation and a Western blot analysis

The cell lysates were prepared in 1% TNE lysis buffer. For immunoprecipitation, the cell lysates were pre-cleared with protein G-Sepharose (for mouse Abs) or protein A-Sepharose (for rabbit Abs) for 1 h at 4°C, and then incubated overnight with either one of the beads that had previously been incubated for 2 h with anti-Flag (Invitrogen), anti-HA (Sigma–Aldrich), anti-c-Myc (Sigma–Aldrich), anti-PRMT5 (Millipore Upstate, Billerica, MA), anti-STAT6 (Santa Cruz Biotechnologies, Santa Cruz, CA) Abs, and anti-GANP mAb (42–23; Kuwahara et al., 2000), or normal mouse or rabbit IgG as a negative control. The immunoprecipitated beads were washed 4 times with TNE lysis buffer. The washed precipitates or whole cell lysates were resuspended in SDS-PAGE sample buffer, boiled for 5 min, analyzed by SDS-PAGE and transferred electrically to the nitrocellulose membrane, which was detected by a Western blot analysis using various kinds of Abs as follows: mouse anti-Flag (Invitrogen), mouse anti-HA, rabbit anti-c-Myc, rabbit anti-STAT6, rabbit anti-phospho-STAT6, mouse anti- β -actin Ab from Sigma–Aldrich, rabbit anti-symmetric dimethylarginine (SYM10 or SYM11), rabbit anti-PRMT5, rabbit anti-JAK3 from Millipore Upstate, rabbit anti-JAK1, rabbit anti-phospho-JAK1 from BioSource, rabbit anti-phospho-JAK3, anti-GANP mAb, and normal mouse or rabbit IgG from Santa Cruz.

2.10. RT-PCR and cDNA cloning

The total RNAs were extracted with TRIzol (Invitrogen), according to the manufacturer's instructions. Random hexamer-primed cDNAs were prepared with Superscript II reverse transcriptase (Invitrogen) and then amplified by PCR with the following primer pairs: *prmt5* (5') (5'-ATGGCGGCGATGGCGGTCTGGG-3') and (3') (5'-TAGAGGCCAATGGTATATGA-3') primers, *prmt1* (5') (5'-ATGGCGGCGAGCCGAGGCC-3') and (3') (5'-TCATCAGCGCATCCGTAGTCG-3') primers, *ganp* (5') (5'-CCGTGGGATGACATCATCAC-3') and (3') (5'-CATGTCCACCATCTCCAGCA-3') primers. The primer pairs of *prmt5* and *prmt1* were also used for the cDNA cloning by adding appropriate restriction enzyme sites at 5'- and 3'-end. The cloning product was once inserted into pBluescript, and the sequence of the inserted fragment was confirmed by sequencing with T7 or T3 primer. Thereafter, the insert was introduced into pcDNA3-HA or -c-Myc to add these tags at the N-termini of the proteins. The tagged cDNAs were further re-cloned into pCXN2 expression vector containing chicken β -globin promoter.

The CSR and ϵ germline transcripts (ϵ GLT) were estimated by RT-PCR (Sugai et al., 2003). A DC-PCR analysis was also performed as described previously (Chu et al., 1992). Specific primers were as follows: V_HJ558 (5'): 5'-CAGGTCCAAGTGCAGCAG-3'; I μ (5'): 5'-CTCTGGCCCTGCTTATTGTTG-3'; C ϵ (3'): 5'-CCAGGTGTCATGGAAGCAGTG-3'; I ϵ (5'): 5'-TGGGAT CAGACGATGGA-GAATAG-3'; GLT (5'): 5'-GCAGAAGATGGCTTCAATAAGAACAGT-3'; and (3'): 5'-TCGTTGAATGATGGAGGATGTGTACGT-3'; nicotinic

acetylcholine receptor β subunit gene (nAChRe) (5') 5'-GGCCGG-TCCACAGCGCGCACTGACACCTAAG and (3') 5'-GCGCCATCGA-TGGACTGCTGTGGGTTTCACCCAG; mouse β -actin (5') (5'-CCTAA-GGCCAACCGTGAAG-3') and (3') (5'-TCTTCATGGTGCTAGGA-GCCA-3'). Semi-quantitative RT-PCR was performed by 5-fold serial dilution of cDNA templates.

2.11. In vitro methyltransferase assay

The pCXN2/*Flag-ganp* or pCXN2/*HA-prmt5* (10 μ g each) was transfected by liposome-mediated transfection (Fugene 6; Roche), into a 100 mm diameter dish of 293T cells. After 48 h culture, the whole cell extract was immunoprecipitated with anti-Flag M2-agarose affinity gel or anti-HA Ab combined with protein A sepharose (Sigma–Aldrich), respectively. The immunoprecipitates were rinsed twice with methylation buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, and 1 mM EGTA) at room temperature, resuspended in 50 μ l of methylation buffer, and 10 μ g of H4, H3, H2A, H2B (Roche) and 1 μ l (0.5 μ M) of S-(5'-Adenosyl)-L-methionine chloride was added. The reactions were carried out at 30°C for 2 h, and then were stopped by the addition of SDS-sample loading buffer. The sample was boiled for 3 min and divided into two aliquots to examine H2A/H4-specific methylation by a Western blot analysis using rabbit polyclonal antibody to histone H2A + H4 (symmetric di methyl R3) Ab (ab5823; Abcam, Cambridge, MA) and to ascertain the immunoprecipitation. The remaining histone in the gel was visualized with Coomassie Brilliant Blue R-250 staining (Nacalai Tesque, Tokyo, Japan).

2.12. Luciferase reporter assay

The 700 bp promoter region of C ϵ was amplified by genomic PCR with 5' proF/E (1+2) (5'-AAAAGATCTACCAGAGTGTG-CAGGTGTGTCTCC-3') for forward primer and 3' proR (5'-AAAAGATCTCCTGTGCAGGCTCCAGGCGTGT-3') for a reverse primer, of the genomic DNA sequence from M12 cells. The PCR product was once cloned into pBluescript vector and sequenced. Then the fragment corresponding to the C ϵ promoter region was excised and inserted into the multiple cloning site of pGVB luciferase reporter cassette in a right direction (TOYO Ink, Tokyo, Japan). The neomycin resistant gene was also inserted into a restriction enzyme site out of multiple cloning sites. Forty μ g of the reporter construct was transfected into the M12 cells by electroporation under the condition of 150 V and 975 μ F. The stable transfectant expressing reporter gene was selected by adding G418 (800 μ g/ml) at 48 h after electroporation. The growing cells were cloned after cell sorting. 2×10^6 of the transfected cells were stimulated with LPS (10 μ g/ml), and with or without mL-4 (100 U/ml) for 18 h.

The reporter construct (4 μ g) was transfected into 90%-confluent COS7 cells in a 6-well culture plate with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The transfectants were selected by adding G418 (600 μ g/ml) at 48 h after transfection. Both or either one of pCXN2/*Flag-ganp* and pCXN2/*HA-prmt5* (3 μ g/each) was transiently transfected into the established COS7 transfectants (1×10^6 cells). After culturing for 6 h, the cells were stimulated with hIL-4 (10 ng/ml) for 18 h. The mock cells were established with the empty vector by adjusting the amount of plasmid DNA. The STAT6-mediated transcription activity was measured as the firefly luciferase activity with a reporter assay kit (Promega).

2.13. Retrovirus transfection

To establish stable transfectants that over-express either PRMT5 or GANP in the reporter cell line, the cDNA with tag was inserted

into the multiple cloning site of MSCV2.2-IRES-GFP retrovirus vector (kindly donated by Dr. Akihiro Kume, Division of Genetic Therapeutics, Center for Molecular Medicine, Jichi Medical University) (Kume et al., 1999). The vector construct was transfected to an EcoPack2-293 packaging cell line (Clontech) with Fugene 6 (Roche) and the supernatant containing infectious virus particles were collected after 48 h. Retrovirus-mediated gene delivery to the M12 reporter cells was performed by repeated spin-inoculation with virus supernatant with 2000 rpm at 33 °C for 1 h. The integration of retrovirus was verified by GFP expression on flowcytometer after 72 h post-infection. The GFP⁺ cells were sorted, and the GFP^{High} clone was selected.

3. Results

3.1. GANP deficient mice show higher serum IgE

B cell-specific conditional targeting of *ganp* gene (deletion of exons 1 and 2) (B-GANP^{-/-}) was carried out previously by a combination of floxed *ganp* mice and *CD19-cre* knockin mice (Kuwahara et al., 2004). The serum Ig classes of the B-GANP^{-/-} mice did not show any decrease or particular increase in the IgM, IgG₁, IgG_{2a}, IgG_{2b}, and IgG₃ classes in comparison to those of the floxed littermates upon either T cell independent (TI)-II or TD Ag. We herein re-examined the serum Igs and found that IgE was significantly elevated in these mice without immunization, regardless of the fact that the other classes of Ig showed no statistically significant difference with comparison to the control littermates as previously observed (Fig. 1), thus implicating that GANP might participate in the suppression of hyper-IgE during immune responses.

3.2. Identification of GANP-associated protein

To screen for GANP-associated protein in cells, we performed *ganp* cDNA transfection into 293T cells, with the expression construct under the promoter of pEF-Bos with 3× Flag-tag and tobacco etch virus (TEV) protease/calmodulin-binding expression construct (Puig et al., 2001). Serial immunoprecipitation with the crude lysate of 1×10^9 cells revealed several protein bands: lane a shows the protein profile bound to the anti-Flag Ab-agarose, lane b as binding with calmodulin beads, and lane c as the eluate (Fig. 2A, upper panel). Several specific bands were purified and their amino acid sequences were determined using the time of flight mass spectrometer (TOF-MAS) system (Fig. 2A, lower panel).

As a GANP-binding protein, we found PRMT5, which is associated with a 50-kDa WD (tryptophan and aspartic acid) repeat protein termed methylosome protein 50 (MEP50) (Friesen et al., 2002). We studied the physical interaction of GANP with PRMT5, in association with MEP50, by a cDNA transfection of COS7 cells. The co-precipitation of Flag-GANP with HA-PRMT5 (left panel) and Myc-MEP50 (right panel) was confirmed (Fig. 2B). Flag-GANP is associated not only with HA-PRMT5, but also with endogenous cellular PRMT5 (Fig. 2C). Another family of arginine methyltransferase, PRMT1, did not bind to GANP (Fig. 2D), thus suggesting that GANP associates with PRMT5 in a specific manner.

To confirm the involvement of arginine methyltransferase, we addressed whether the GANP immunoprecipitates contained arginine methylation activity, using an *in vitro* assay with histones H2A, H3, and H4 as the exogenous substrates. The anti-Flag-GANP immunoprecipitate showed arginine methylation on H2A, and more distinctly on H4, similar to that with the anti-HA-PRMT5 immunoprecipitate, by a Western blot analysis (Fig. 2E). These

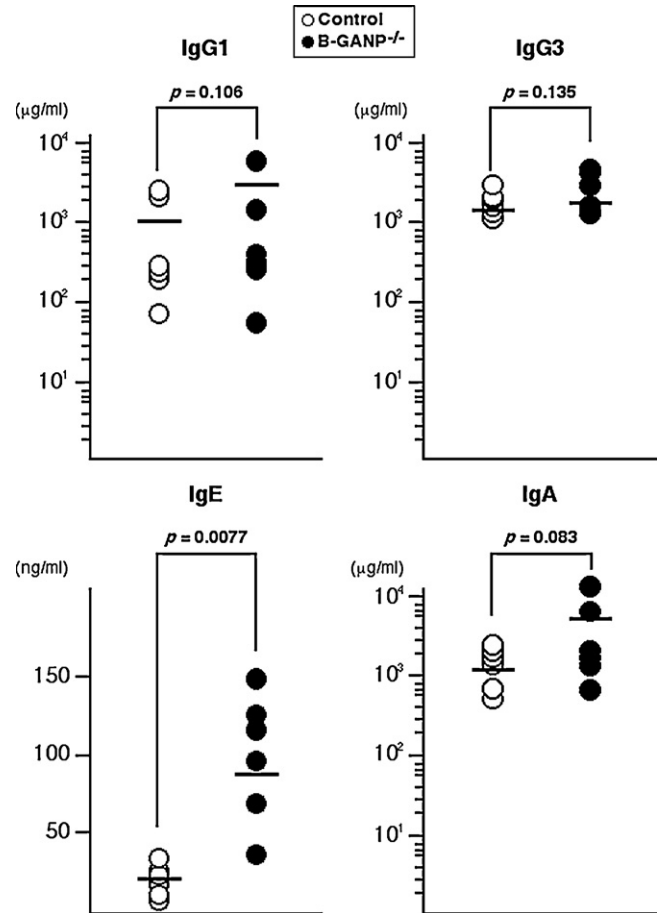


Fig. 1. High serum IgE in B-GANP deficient mice. Concentrations of IgG₁, IgG₃, IgE, and IgA were measured in the sera of non-immunized control (○) and B-GANP^{-/-} (●) mice by ELISA. Six mice were examined per group and the result is shown as the mean ± SEM with P-value measured by Student's t-test.

results imply that GANP is physically associated with JAK-binding arginine methyltransferase PRMT5 in cells.

3.3. Expression of PRMT5 in B cells and mature GC B cells

Prmt1 transcript is detected ubiquitously in various organs and tissues, but *prmt5* transcription is detected selectively in the heart, lung, and muscle (Fig. 3A). *Prmt5* transcription was not detected in the spleen, thymus, or Peyer's patches, except for purified B cells. Naïve B cells in the spleen showed only a low level of *prmt5* transcripts; however, the stimulation of B cells *in vitro* with LPS induced a marked increase after 12 h, which then disappeared rapidly until 24 h, and the addition of IL-4 prolonged *prmt5* transcription until 24 h (Fig. 3B). *Ganp* transcription was also up-regulated at 24 and 48 h after stimulation (more than the basal level in naïve B cells). Stimulation with anti-IgM and anti-CD40 Abs also increased *prmt5* transcription (Fig. 3C, left panel). The GC B cells expressing B220 and GL7 were purified after immunization with TD-Ag sheep red blood cell (SRBC). The transcription of *prmt5* increased in the GC B cell population, similar to that for the *ganp* transcript (Fig. 3C, right panel). The arginine-methylated endogenous proteins detected by a Western blot analysis increased markedly in the B cells stimulated with anti-IgM and anti-CD40 Abs (Fig. 3D, left panel), and also in the GC B cells (B220⁺GL7⁺) purified from the spleen immunized with SRBC (Fig. 3D, right panel).

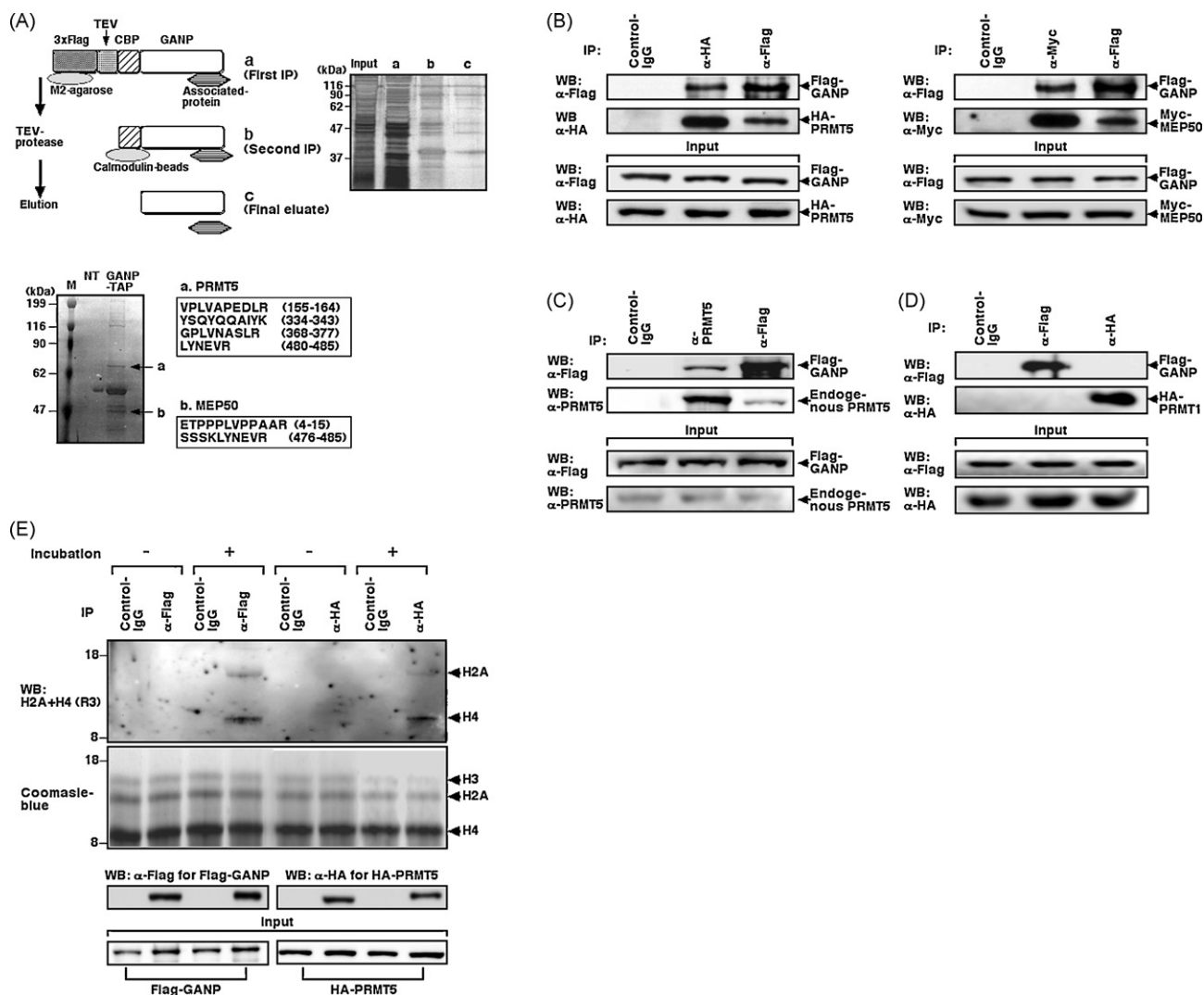


Fig. 2. Identification of GANP-associated molecules. (A) Upper left panel: the recombinant GANP protein includes two different tags, Flag and calmodulin-binding protein (CBP), as described in Section 2. Upper right panel: the immunoprecipitate with anti-Flag (M2) beads (a), calmodulin beads (b), or final eluate (c) was resolved on SDS-PAGE with silver staining. Lower panel: the final eluate was resolved on SDS-PAGE with Simply Blue dye. The proteins at the visible bands were extracted, and analyzed by the peptide mass finger print method. The tryptic peptide sequences of PRMT5 and MEP50 identified were included in the tables, and the numbers in parentheses correspond to the amino acid position. NT: negative control from *mock* transfectant. (B) The association of PRMT5 and MEP50 with GANP were analyzed by co-transfection of COS7 cells with *Flag-ganp*, along with *HA-prmt5* or *Myc-mep50* vectors, as indicated. The lysates were immunoprecipitated (IP) with anti-Flag (α -Flag), anti-*HA* (α -HA), and anti-Myc (α -Myc) Abs, and Western blot analysis (WB) was performed as indicated. (C) The association of endogenous PRMT5 with the Flag-tagged GANP protein was examined by transfection of *Flag-ganp* cDNA in 293T cells. The Flag-tagged GANP-immunoprecipitate was analyzed by Western blotting with anti-PRMT5 (α -PRMT5) Ab, and the PRMT5 immunoprecipitate was blotted with α -Flag Ab. (D) The COS7 cells were transiently transfected with the *Flag-ganp* and *HA-prmt1* vectors. The whole-cell extracts were immunoprecipitated with α -Flag or α -HA Ab, and a Western blot analysis was conducted as indicated. (E) The 293T cells were transfected with the *HA-prmt5* or *Flag-ganp* vector and the whole-cell extracts were immunoprecipitated with α -HA or α -Flag Ab. An *in vitro* methylation assay was conducted with the immunoprecipitates and pooled histones. The top panel, methylated histones were visualized with the anti-symmetrical dimethylated histone Ab [H2A + H4 (R3)]; second panel, the corresponding Coomassie staining of SDS-PAGE; third panel, analyzed by a Western blot analysis with α -Flag or α -HA Ab; bottom panel, amount of Flag-GANP or HA-PRMT5 in the input was determined with α -Flag or α -HA Ab. All transfection experiments were repeated more than twice and representative one was shown.

3.4. Regulatory role of GANP in the signal transduction pathway of the JAK/STAT complex associated with PRMT5

The previous report demonstrated the association of PRMT5 with JAK1 and JAK3 as a JAK-binding arginine methyltransferase, while the down-stream signaling pathway has not been proven yet (Pollack et al., 1999). We then studied whether GANP forms a complex with JAKs associated with PRMT5. Flag-GANP was co-precipitated with Myc-JAK1, and Myc-JAK1 was reversibly co-precipitated with Flag-GANP from the transfectants (Fig. 4A, left panel). As a negative control, Flag-GANP was not co-precipitated with Myc-EGFP (Fig. 4A, right panel). Flag-GANP was co-precipitated simultaneously with HA-PRMT5 and endoge-

nous JAK1 (Fig. 4B). Myc-JAK3 was clearly, but less intensely, co-precipitated with Flag-GANP (Fig. 4C). These results indicate that GANP is associated with PRMT5 and JAKs in cells. We examined the association of GANP and PRMT5 as the endogenous proteins in the mouse germinal center B lymphoma cell line M12 that expressed GANP at the high level (Fig. 4D). PRMT5 was clearly co-precipitated with GANP, JAK1 and JAK3 (lane 3). As the reversal co-precipitation, GANP was co-precipitation with PRMT5. The 210-kDa GANP was not strongly but clearly detected in the co-precipitate of PRMT5 that bound strongly with JAK1 and at the lesser degree with JAK3 (lane 2).

We next analyzed how the JAK/STAT signal transduction pathway is regulated by GANP in B cells. Activation of JAK/STAT signal

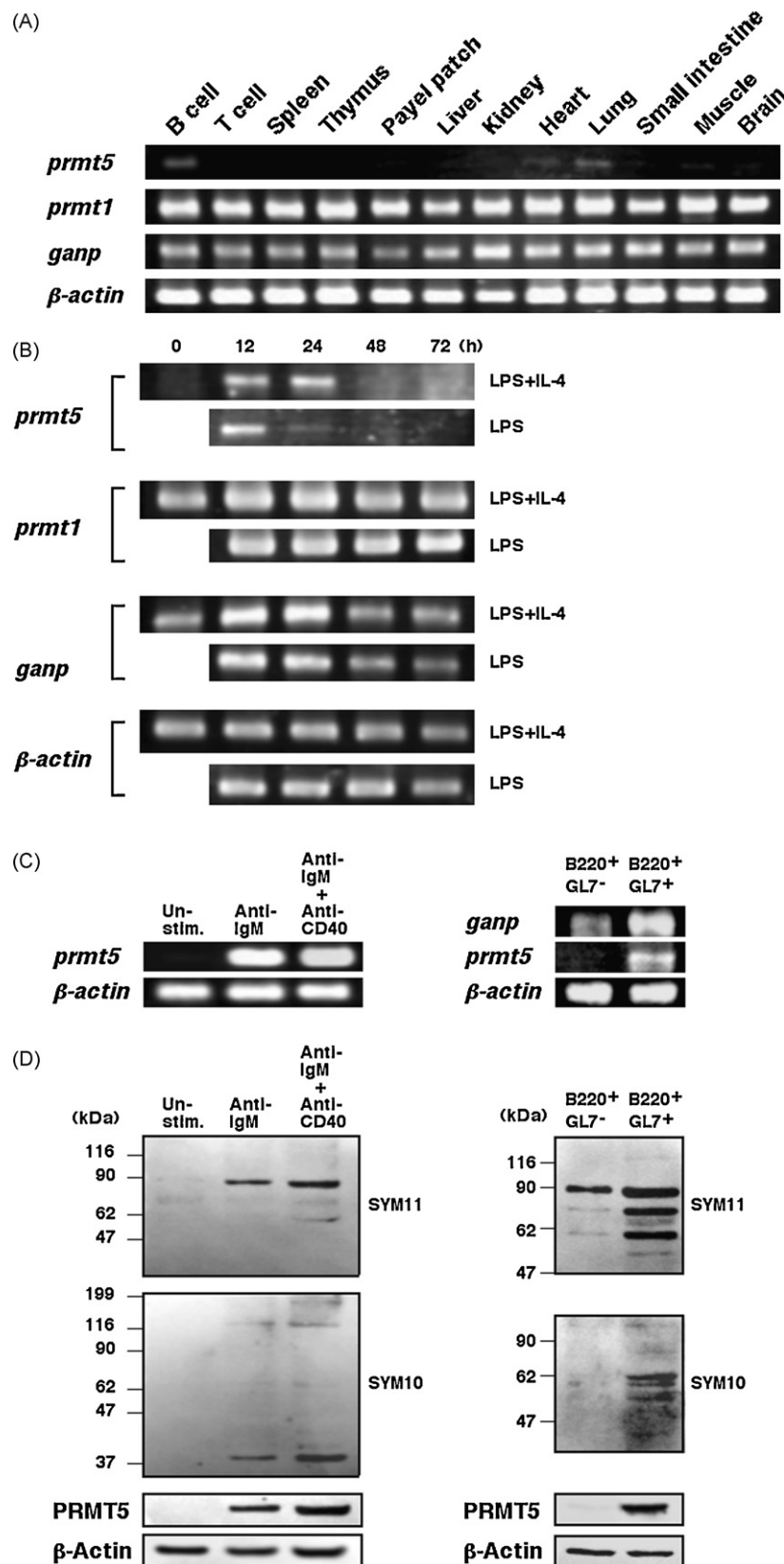


Fig. 3. Inducible expression of PRMT5 in activated B cells. (A) The expression of *prmt5*, *prmt1*, and *ganp* transcripts in various organs or cells. The total RNA was isolated from the tissues or organs listed, and RT-PCR was conducted with the gene-specific primers described in Section 2. The amplification of β -actin was an internal control. (B) Purified splenic B cells were stimulated with LPS or LPS plus IL-4. RT-PCR was performed with total RNA, using gene-specific primers to examine the inducible transcription of *prmt5*, *prmt1*, and *ganp*. The amplification of β -actin was an internal control. (C) Left panel, splenic B cells were stimulated with anti-IgM or anti-IgM plus anti-CD40 Abs for 48 h. The inducible expression of *prmt5* was examined by RT-PCR with gene-specific primers, using cDNAs from unstimulated (Unstim.) or stimulated samples, in comparison with the β -actin controls. Right panel, B cells were isolated from the spleens of C57BL/6 mice at 12 d post immunization with SRBCs, and were stained with APC-anti-B220

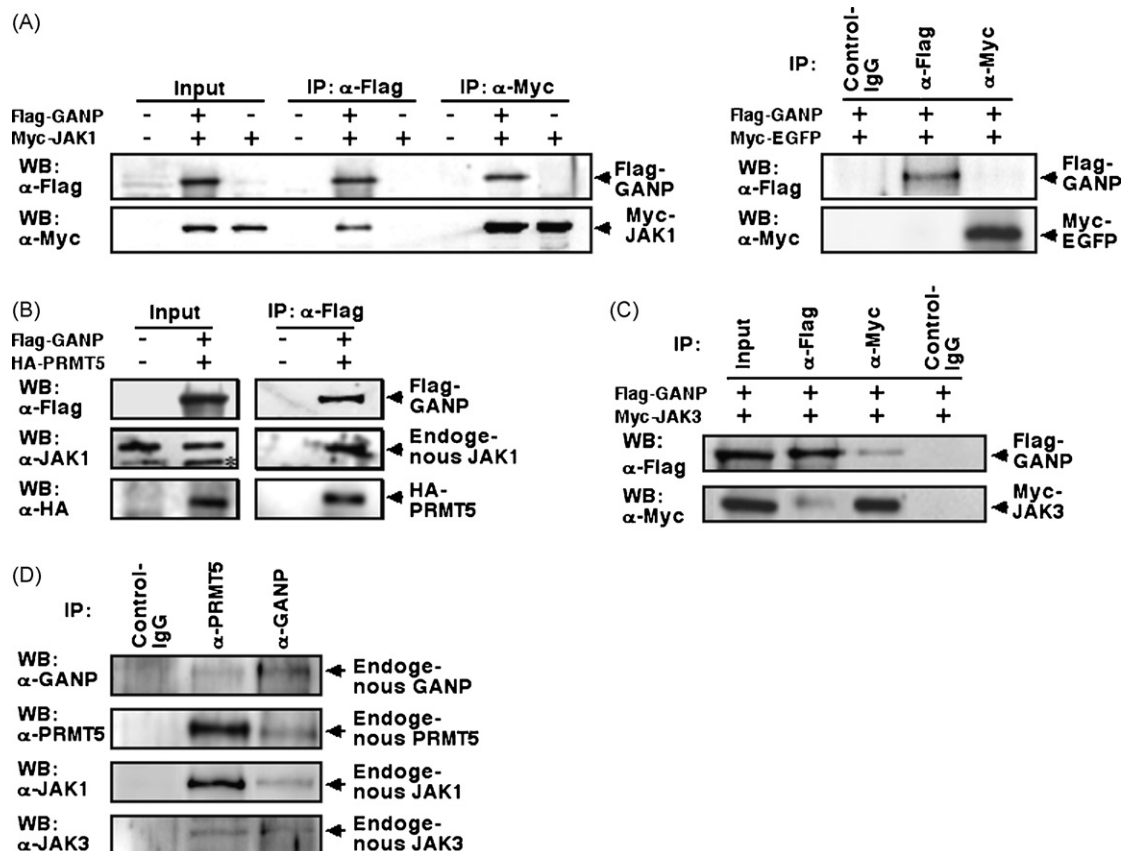


Fig. 4. GANP associates with JAKs that are involved in IL-4 signaling. (A) The association of JAK1 with GANP was analyzed by transient co-transfection of COS7 cells with *Flag-ganp* and *Myc-jak1* vectors. The cell lysates were immunoprecipitated (IP) with α -Flag or α -Myc Abs, and a Western blot analysis (WB) was performed reciprocally. The right panel showed the negative control with Myc-EGFP. (B) HepG2, a human hepatoma cell line with a high level of endogenous JAK1, was transfected with *Flag-ganp* and *HA-prmt5* vectors. The cell extracts were immunoprecipitated with α -Flag Ab and a Western blot analysis was performed. Asterisk (*) indicates the degraded JAK1. (C) The association of JAK3 with GANP was analyzed by co-transfection of the *Flag-ganp* and *Myc-jak3* vectors into COS7. The cell lysates were immunoprecipitated with α -Flag and α -Myc Abs, and analyzed as indicated. (D) The association of GANP and PRMT5 was examined as the endogenous proteins in the M12 B cell line. Immunoprecipitation and Western blot analysis were carried out with the specific antibodies against GANP, PRMT5, JAK1 and JAK3 as described in Section 2. All transfection experiments were repeated more than twice and representative one was shown.

transduction was examined after stimulation of B-GANP^{-/-} with LPS and IL-4. GANP^{-/-} B cells showed an increase in phosphorylation of JAK1, and a marked increase in phosphorylation of STAT6 in comparison to that of the control cells after stimulation (Fig. 5A, left panel). In contrast, B cells from *ganp* cDNA transgenic mouse (GANP^{TC}) (Fujimura et al., 2003; Sakaguchi et al., 2005) showed decreases in phosphorylation of JAK1, JAK3 and STAT6 after stimulation with LPS and IL-4 (Fig. 5A, right panel). IL-4 and LPS induced remarkable arginine methylation of STAT6 similar to STAT6 phosphorylation in B-GANP^{-/-} B cells in comparison to the control B cells (Fig. 5B). These results suggest that GANP negatively regulates STAT6 activation in B cells, by down-regulating the arginine methyltransferase activity of PRMT5 involved in STAT6 activation through arginine methylation.

3.5. Regulation of STAT6 transcription activity by GANP in B cells

To confirm the regulation of the JAK/STAT signal transduction pathway in B cells, we transfected *ganp* or *prmt5* cDNA into M12 cells. M12 cells induced *germ line* transcripts for *Cε* (*εGLT*), necessary for CSR in response to stimulation with LPS and IL-4 for 24 h

(Fig. 6A). The same stimulation induced phosphorylation of STAT6 at 15 min after the addition of IL-4 (Fig. 6B). Arginine methylation on activated STAT6 was clearly detected in IL-4 stimulated M12 cells with anti-dimethylarginine specific Ab (Fig. 6C). We examined the effect of GANP and PRMT5 upon the *εGLT* transcription in M12 cells (Fig. 6D). GANP suppressed *εGLT* transcription induced by the stimulation with LPS and IL-4 in M12 cells, but PRMT5 augmented the *εGLT* transcription.

In order to quantitatively evaluate the effect of GANP on IL-4-mediated JAK1 and JAK3/STAT6-signaling, STAT6 activation was measured in M12 cells by the luciferase reporter construct under the *mouse promoter* of *Cε* carrying STAT6-binding motifs. M12 cells showed a marked transcription of the luciferase reporter gene after stimulation with IL-4. The transfection of *ganp* cDNA down-regulated the IL-4-induced luciferase reporter activity to ~60% of the *mock* transfected control cells (Fig. 6E), while *prmt5* cDNA markedly up-regulated the luciferase transcription activity, thus indicating that the IL-4-induced, STAT6-mediated gene transcription is regulated by arginine methyltransferase PRMT5 in B cells. To confirm the inhibitory effect of GANP on the PRMT5-induced STAT6 activation, we measured the STAT6-mediated gene tran-

and FITC-anti-GL7 Abs, and sorted into two populations, B220⁺GL7⁺ GC B cells or B220⁺GL7⁻ non-GC B cells. The transcription of *prmt5* and *ganp* was examined by RT-PCR. (D) The B cells stimulated or sorted in (C) were lysed with TNE buffer, and then subjected to a Western blot analysis with anti-symmetrically dimethylarginine Abs (SYM10 and SYM11) or with anti-PRMT5 Ab. The amount of β -actin was a loading control.

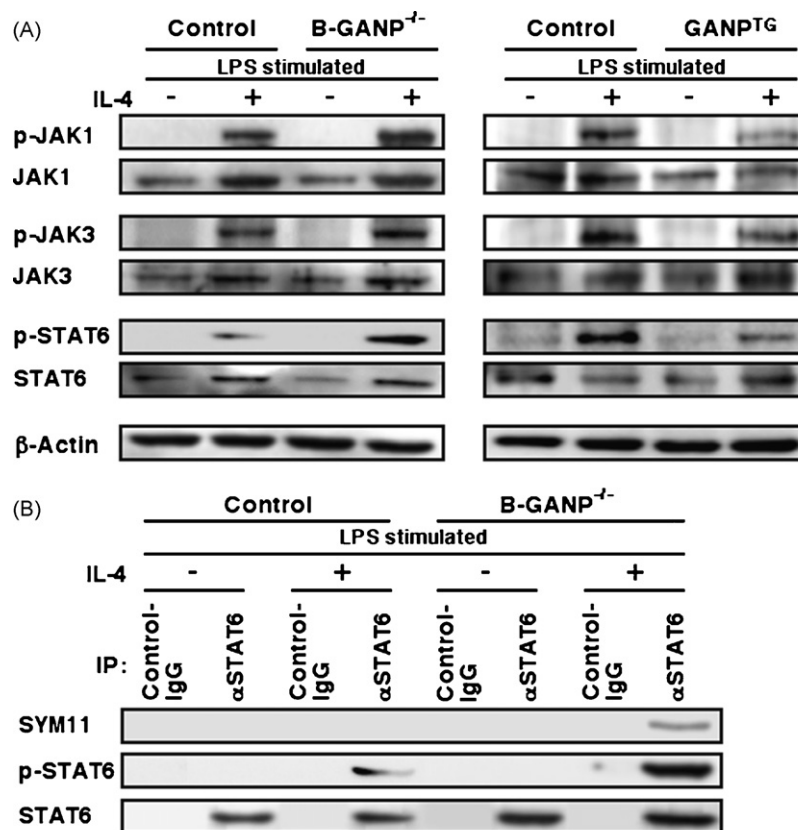


Fig. 5. GANP showed a negative effect on JAKs/STAT6-signaling pathway. (A) The phosphorylation status of JAK1, JAK3, and STAT6 was examined in B cells from C57/BL6, GANP-deficient B cells from B-GANP^{-/-}, and GANP over-expressing B cells from GANP^{TG} mice. Splenic B cells were pretreated with LPS (10 μg/ml) for 16 h, and then stimulated with mIL-4 (100 U/ml) for 15 min. The whole-cell extracts from transfectants were subjected to SDS-PAGE, and a Western blot analysis was performed with Abs of both non-phosphorylated and phosphorylated forms of JAK1, JAK3, STAT6, and SYM11. β-Actin was a loading control. (B) Splenic B cells from the control or B-GANP^{-/-} mice were stimulated with LPS and IL-4 and subjected to anti-STAT6 (α-STAT6) immunoprecipitation, and then Western blotting analyses were carried out with SYM11, anti-phospho-STAT6 (α-p-STAT6), and α-STAT6 Abs. All experiments were repeated twice and representative one was shown.

scription activity after co-transfection of *ganp* and *prmt5*. Here, we used the IL-4-responsive COS7 cells (Moriggi et al., 1997; Carlsson et al., 2006) that express endogenous STAT6 (data not shown) and carry the STAT6/Luc reporter construct. The over-expression of *ganp* suppressed the enhancement of STAT6 activity caused by *prmt5*, thus suggesting that GANP down-regulates PRMT5-associated JAK-STAT-signaling down-stream of IL-4 receptor (Fig. 6F).

In vitro stimulation of GANP^{-/-} B cells with LPS and IL-4 induced the enhancement of *εGLT* transcription in comparison with control B cells, revealing the suppressive function of GANP on IgE production in normal B cells (Fig. 6G). Finally, we examined whether class switching in the GC B cells of B-GANP^{-/-} mice is affected during the immune responses *in vivo*. GC B cells were defined by staining with a pan-B cell marker B220 and the germinal center B cell marker of GL7. RT-PCR using the 5'-primer for *V_H558* or the *Iμ* region and the 3'-primers corresponding to *Cε*-region shows the class switching events after immunization with Ag in the adjuvant alum (Cunningham et al., 2004). B-GANP^{-/-} GC B cells showed increased transcription of *ε* (*V_H558-Cε* and *Iμ-Cε*) in comparison to control GC B cells (Fig. 6G, left panel). Digestion-circularization (DC-PCR) showed an increased intensity at the band of *Cε*-switching in B-GANP^{-/-} GC B cells (Fig. 6G, right panel). The results confirmed that GANP down-regulates JAK1- and JAK3-mediated STAT6 signal transduction in GC B cells, through a mechanism associated with arginine methyltransferase PRMT5, and suppresses hyper-IgE during immune responses.

4. Discussion

We herein provide evidence regarding the down-regulation of IL-4-mediated signaling in GC B cells through regulation of arginine methyltransferase activity. GANP was found to be associated with arginine methyltransferase PRMT5, which was in a complex with JAK1 and JAK3. GANP deficiency caused increased STAT6 activity in response to IL-4 stimulation. The results suggest that GANP negatively regulates the IL-4-induced JAK1 and JAK3/STAT6-mediated gene activation of various molecules, including AID and *Cε* germ line transcription required in GC B cells.

The protein modification with arginine methylation plays a role in various biological functions; including RNA processing, signal transduction, transcriptional regulation, and DNA repair (Bedford and Richard, 2005; Lake and Bedford, 2007; Bachand, 2007). PRMT5 was identified as a JAK-binding arginine methyltransferase in association with MEP50, a molecule involved in RNA splicing complexes (Pollack et al., 1999; Friesen et al., 2002). We have not neglected the substrates other than those of the JAK/STAT-mediated signal transduction pathways; however, the results clearly demonstrated that GANP affects the IL-4-mediated gene activation in GC B cells through the interaction with PRMT5.

GANP is induced by co-ligation of BCR and CD40 in B cells. In GCs, B cells are presumably stimulated variously at different stages through multiple co-stimulatory molecules, in addition to BCR ligation. GANP induced by CD40 ligation may regulate other

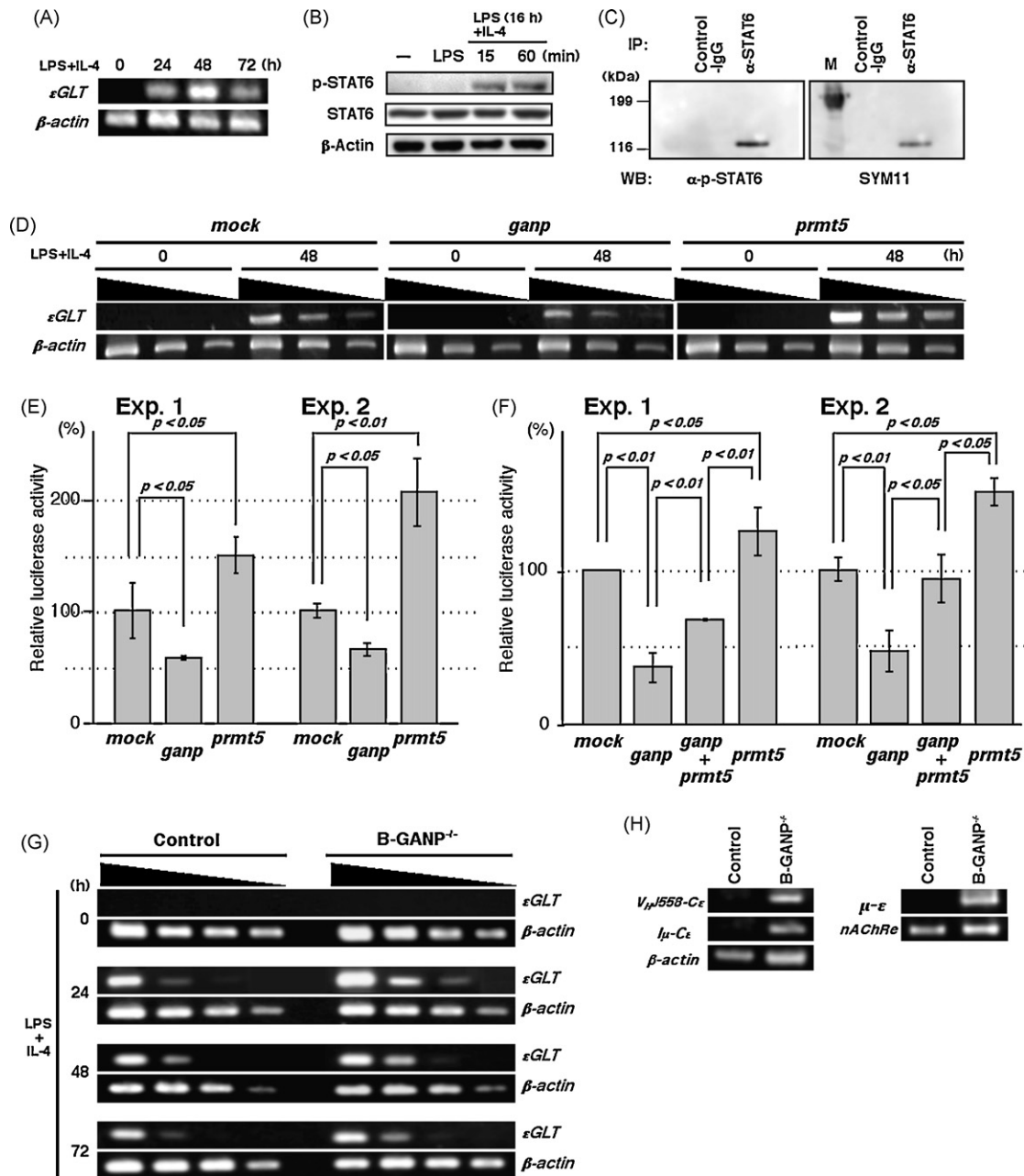


Fig. 6. GANP down-regulates STAT6 activation in B cells. (A) M12 cells were cultured with LPS and IL-4. RT-PCR was performed with the total RNA purified from the cells using the primers for ϵ GLT. (B) The activation of STAT6 was examined in M12 cells pre-cultured with LPS (10 μ g/ml) for 16 h. The phosphorylation of STAT6 was analyzed after the addition of mL-4 (100 U/ml) by a Western blot analysis with α -p-STAT6 and α -STAT6 Abs. β -Actin was a loading control. (C) M12 cells were pre-cultured with LPS (10 μ g/ml) for 16 h and stimulated with mL-4 (100 U/ml) for 15 min. The cell extracts were prepared with TNE lysis buffer and immunoprecipitated with α -STAT6 Ab or control rabbit IgG. A Western blot analysis was performed with SYM11, and the blot was re-probed with α -p-STAT6 Ab. M: Molecular weight marker. The band around 200 kDa in the marker lane is myosin which is constitutively methylated on arginine residues symmetrically. (D) ϵ GLT was estimated by RT-PCR described above using cDNA prepared from the stable *mock*, *ganp*, and *prmt5* transfectants of M12. RT-PCR was performed with 5-fold serially diluted cDNA from unstimulated or stimulated cells. (E) The IL-4-mediated STAT6 activation was measured by the reporter assay. M12 cells were first stably transfected with STAT6/Luc reporter to measure the transcription activity, and then *Flag-ganp* or *HA-prmt5* cDNA was transfected. The transfected cells were cultured with LPS and mL-4 for 18 h. The STAT6 activity was assessed by measuring the production of luciferase in the cell extracts in triplicate, and shown as the mean $\pm$ SEM with P-values measured by Student's *t*-test. The relative luciferase activity was represented based on the activity of *mock* transfection being defined as 100%. Two independent assays were shown. (F) The effect of *ganp* on STAT6 activity was confirmed in the COS7 reporter system. The COS7 STAT6/Luc reporter stable transfectant was established in advance and the expression constructs of *ganp* or *prmt5* were transiently transfected. The transfectants were stimulated with hIL-4 for 18 h before measuring the STAT6 activation. The STAT6 activity was assessed as described in (E). Two independent assays were shown. (G) ϵ GLT was estimated by RT-PCR described above using cDNA prepared from the control and B-GANP^{-/-} mice. RT-PCR was performed with 5-fold serially diluted cDNA from unstimulated or stimulated cells. (H) GC B cells defined as B220⁺GL7⁺ were isolated from (4-hydroxy-3-nitrophenyl) acetyl-chicken gamma-globulin (NP-CG) challenged control or B-GANP^{-/-} mice at day 9 post immunization by cell sorting. The total RNA was purified from these cells and used for RT-PCR to examine class switch recombination (left panel). The amplification of β -actin was performed as an internal control. Genomic DNA was also purified from the same population and the genome-based class switch recombination was examined by DC-PCR (right panel). The amplification of nAChRe (nicotinic acetylcholine receptor β subunit gene) was performed as an internal control.

signals, including IL-4 stimulation, through arginine methyltransferase PRMT5, while the possibility of direct interaction of GANP in the complex of transcription co-regulators of STAT6 remains to be clarified. STAT6 has been shown to interact with other regulators including NF- κ B (Messner et al., 1997; Shen and Stavnezer, 1998; Warren et al., 1999), cAMP response element binding protein (CREB)-binding protein (CBP/p300), nuclear coactivator 1 (NcoA-1), a CREB-binding-protein-associated member of the p160/steroid receptor coactivator family (Razeto et al., 2004), and glucocorticoid receptor (Biola et al., 2000), thus suggesting that GANP participates in the complex with either of these molecules and regulates transcription through arginine methylation. The methylation of Arg-27 also modulates STAT6 phosphorylation and nuclear translocation, and DNA binding (Chen et al., 2004).

Previous studies have demonstrated the function of GANP in affinity maturation of GC B cells upon immunization with TD-Ag (Kuwahara et al., 2004; Sakaguchi et al., 2005). The function of GANP may be explained with the following model: for the affinity maturation of B cells against the specific Ag, naïve B cells need to undergo SHM of *Ig V-region* genes and *C-region* CSR at the centroblast stage. The induction of AID by the stimulation with Ag, IL-4, and the CD40/CD40L interaction is indispensable for these events in the GC region. IL-4 stimulation induces the activation of JAK1 and JAK3 in B cells, thus leading to the phosphorylation of STAT6 within minutes to hours, and the activated STAT6 forms dimers and is translocated to the nucleus to access the STAT6-binding DNA elements. Simultaneously, Ag stimulation induces PRMT5 within 24–48 h, and PRMT5 protein associates with JAKs to re-activate STAT6 either in the cytoplasm or the nucleus. The positive regulation of STAT6-signaling induces AID expression in association with NF- κ B signaling, which is mediated by CD40 stimulation (Zhou et al., 2003). GANP is also induced for 24–48 h after Ag stimulation with BCR and CD40-mediated signaling in GC B cells. The results demonstrated that JAK/STAT signaling leading to class switching induced by AID is augmented by the arginine methylation of STAT6 through the interaction with PRMT5, whereas GANP antagonizes this pathway to maintain the threshold of STAT6 activation for proper class switching in antigen stimulated GC B cells.

PRMT5 is also associated with other molecules such as RNA polymerase II, FCP1 phosphatase, or Blimp-1; however, it is not clear how this mutual association regulates molecular function (Amente et al., 2005; Ancelin et al., 2006). The binding of GANP to PRMT5 presumably down-regulates the arginine methyltransferase activity of PRMT5, and suppresses STAT6 activation, thus leading to a decrease in STAT6-mediated AID expression. The limitation of AID expression might be necessary for the maintenance of BCR specificity. B cells with high-affinity BCRs will leave the GCs from the follicular dendritic cell network, and migrate to the appropriate site for an immune response, at which time B cells need to proliferate and differentiate without changing the affinity and *V-region* specificity, as occurs in long-term immune responses.

The class switching to IgG₁ also requires IL-4-induced STAT6 activation, however, B-GANP^{-/-} mice showed a selective increase of IgE class switching. While the class switching to both IgG₁ and IgE are induced through activation of STAT6, the promoters for ϵ and γ 1 transcription might have different sensitivities to the nuclear transcription factor STAT6. Mao and Stavnezer (2001) reported that the relative affinities of the ϵ and the γ 1 *Stat6*-binding sites have a 10-fold difference in the competition assay by EMSA. Therefore, the negative regulatory activity through GANP/PRMT5 upon JAK-STAT6-signaling may appear more markedly on IgE class switching.

Collectively, GANP plays a role in suppression of the hyper-IgE trend of the individual and maintains the production of IgG class Ab against TD-Ags under physiological immune responses. Further studies of the B cell endogenous suppressive pathway of IgE class

switching would be important to elucidate the details of allergic diathesis.

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