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Gp49B is a pathogenic marker for autoantibody-producing plasma cells in lupus-prone BXSB/*Yaa* mice

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Abstract

Immune homeostasis is critically regulated by the balance between activating and inhibitory receptors expressed on various immune cells such as T and B lymphocytes, and myeloid cells. The inhibitory receptors play a fundamental role in the immune-checkpoint pathway, thus maintaining peripheral tolerance. We recently found that expression of leukocyte immunoglobulin-like receptor (LILR)B4, an inhibitory member of the human LILR family, is augmented in autoantibody-producing plasmablasts/plasma cells of systemic lupus erythematosus (SLE) patients. However, the mechanism behind the "paradoxical" upregulation of this inhibitory receptor upon pathogenic antibody-secreting cells is yet to be known. To this end, in this study we examined if glycoprotein 49B (gp49B), the murine counterpart of human LILRB4, is also elevated in autoantibody-producing cells in several SLE mouse models, and tried to clarify the underlying mechanism. We found that gp49B is expressed on plasma cells of lupus-prone models but not of healthy C57BL/6 mice, and the level was positively correlated to the anti-double-stranded DNA IgG titer in serum. Gp49B genetic deletion, however, did not abolish the serum autoantibodies or fully ameliorate the lethal glomerulonephritis, indicating that gp49B is not the sole regulator of lupus but a pathogenic element in the disease. We conclude that the elevated expression of this inhibitory receptor on pathogenic plasma cells was also relevant upon the murine SLE model. The mechanism of gp49B underlying the disease progression in lupus-prone mice has been discussed.

Introduction

Inhibitory receptors play an important part in the immune checkpoint pathway, because achieving a balance between activation and inhibitory actions in the immune system is crucial for effective immune responses to pathogens, and simultaneous protection of self from autoimmune responses (1). Most inhibitory receptors exert inhibitory actions through immunoreceptor tyrosine-based inhibitory motifs (ITIMs) in their cytoplasmic tails to recruit SH2-domain containing tyrosine phosphatases: SHP-1 and SHP-2 or lipid phosphatases: SHIP-1 and SHIP-2 (1, 2). In T lymphocytes, the co-inhibitory receptors CTLA-4 and PD-1 are pivotal regulators for the initial and later stages of T cell activation, respectively (3, 4). Depletion of CTLA-4 and/or PD-1 perturbs the regulatory T cell function to suppress T cell priming and to maintain tolerance, resulting in murine experimental autoimmune thyroiditis or type I diabetes (5-7). In B lymphocytes, the loss of counterbalancing effects of inhibitory receptors such as FcγRIIb, CD22 and Siglec-G can lead to the development of autoimmune diseases like systemic lupus erythematosus (SLE) involving the production of a series of anti-nuclear autoantibodies such as those against double-stranded DNA (dsDNA) (1, 8). Disease susceptibility in the majority of SLE mouse models (NZB, BXSB, SB/Le and MRL) (9), as well as SLE patients (10, 11), is associated with polymorphisms of *FCGR2B*, which encodes FcγRIIb. In support of this, we reported that the genetic combination of $FCGR2B^{-/-}$ and $SLAM^{129}$ led C57BL/6 (B6) mice prone to glomerulonephritis with the elevation of the serum level of anti-nuclear autoantibodies (12).

Murine glycoprotein 49B (gp49B), a 49-kDa membrane protein, is another inhibitory receptor with two ITIMs in its cytoplasmic tail. It belongs to the immunoglobulin (Ig) superfamily with another family member, gp49A, that harbors no specific signaling motifs (13-15). Gp49B was first reported regarding its expression and inhibitory action on bone

marrow-derived mast cells (BMMCs) (14, 16). When co-ligated with FcεRI on BMMCs, gp49B inhibits mast cell exocytosis by recruiting SHP-1 (16, 17). The suppressive function of gp49B in innate and adaptive inflammation is executed by neutrophils (18) and mast cells (19), respectively. In our previous study, gp49B on dendritic cells was found to play a suppressive role in preventing excessive activation of T cells *in vitro* and *in vivo* (20). Among naïve cells in the hematopoietic system, gp49B is reported to be expressed only on Mac-1^+ and Gr-1^+ myeloid cells. However, expression of gp49B can be induced upon *in vitro* stimulation or *in vivo* infection. For instance, upon exposure to murine cytomegalovirus or *Listeria monocytogenes*, gp49B is expressed on natural killer cells, and then suppresses cytokine release (21). In the B cell lineage, gp49B was discovered on mature marginal zone B (MZB) cells and memory B cells, but not on naïve B cells. It exerts an inhibitory effect on the differentiation of MZB cells and memory B cells into plasmablasts or plasma cells, and subsequent antibody production (22).

The human counterpart of gp49B is considered to be leukocyte Ig-like receptor (LILR)B4 (B4, also known as ILT3 or CD85k) based on their syntenic chromosomal locations and structural similarities (23, 24). B4, together with other LILRB members, is an inhibitory isoform of the LILR family that negatively regulates immune responses via tolerized antigen-presenting cells (25). Differing from mouse gp49B, B4 is not expressed on memory B cells, but on plasmablasts of healthy individuals (26). Interestingly, the expression of B4 was found to be increased on plasmablasts/plasma cells from SLE patients (27), and these $B4^{high}$ cells contained abundant anti-dsDNA Ig V_H transcripts, suggesting a contradictory role of this inhibitory receptor as a pathogenic marker. However, the link between elevated B4 expression and autoantibody production has yet to be elucidated.

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In this study, we aimed at clarifying if murine gp49B expression is indeed upregulated on plasma cells of SLE model mice, as in human SLE patients, and if so, attempted to elucidate the pathogenic role of the augmented gp49B expression in autoimmunity such as in autoantibody production and glomerulonephritis. We found that lupus-prone mice, but not healthy strain C57BL/6 ones, exhibited gp49B expression on their plasma cells. The genetic deficiency of gp49B tended to slightly ameliorate SLE disease in BXSB/Y chromosome-linked autoimmune accelerator (*Yaa*) mice, because the anti-dsDNA IgG level in serum was reduced, survival rate mildly improved, and PAS-positive deposition in the kidneys was reduced, although this genetic defect did not abolish autoantibodies in serum or eliminate the antibody-secreting cells in spleens from BXSB/*Yaa* mice. These results suggest that gp49B paradoxically plays a pathogenic role in autoantibody production and glomerulonephritis.

Materials and Methods

Mice

C57BL/6 (B6) and non-obese diabetic NOD/ShiJcl (NOD) mice were purchased from CLEA Japan (Kawasaki, Japan). BXSB/MpJJmsSlc- *Yaa* (BXSB/*Yaa*), MRL/MpJJmsSlc-*lpr*/*lpr* (MRL/*lpr*) and Slc:NZBWF1((NZB × NZW) F_1) mice were purchased from Japan SLC (Shizuoka, Japan). FcγRIIB (RIIB)-deficient mice with the B6 background excepting 129-derived *SLAM* locus (RIIB$^{-/-}$*SLAM*^{129}) (28) and gp49B-deficient mice (gp49B$^{-/-}$) with the B6 background (20) were used. Gp49B-deficient RIIB$^{-/-}$*SLAM*^{129} mice (gp49B$^{-/-}$RIIB$^{-/-}$*SLAM*^{129}) were generated by crossing gp49B$^{-/-}$ mice with RIIB$^{-/-}$*SLAM*^{129} mice. Gp49B-deficient BXSB/*Yaa* mice (gp49B$^{-/-}$BXSB/*Yaa*) were generated by crossing gp49B$^{-/-}$ mice with BXSB/*Yaa* mice for at least 7 generations. All mice were maintained and

bred in the animal facility of the Institute of Development, Aging and Cancer, Tohoku University, under specific pathogen-free conditions. All animal protocols were reviewed and approved by the Animal Studies Committee of Tohoku University.

Flow cytometry analysis and cell sorting

Single cell suspensions were prepared from spleens and bone marrow after red blood cell depletion by ammonium chloride lysis. Cells were stained for 30 min on ice in phosphate-buffered saline (PBS) containing 2% fetal calf serum (FCS) with different combinations of the following antibodies conjugated with FITC or Alexa Fluor® 488, PE, PerCP-Cy5.5, APC or Alexa Fluor® 647, Pacific Blue™, or biotin: anti-mouse CD21/CD35 (BD Biosciences, San Jose, CA, USA), anti-mouse CD11b, anti-mouse CD19, anti-mouse CD23, anti-mouse/human CD45R/B220, anti-mouse CD85k (gp49 receptor) and anti-mouse CD138 (Syndecan-1) (Biolegend, San Diego, CA, USA). Samples were analyzed and sorted using FACSAria III (BD Biosciences). Data were collected using FACS Diva software (BD Biosciences) and analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA). Cells were analyzed or sorted based on the following gating: plasma cells ($B220^{-}CD138^{high}$), follicular B cells ($CD19^{+}CD21^{+}CD23^{high}$), marginal zone B cells ($CD19^{+}CD21^{high}CD23^{low}$), and monocytes ($B220^{-}CD11b^{+}$). Sorted cells were suspended in RPMI 1640 medium supplemented with 10% FCS, 1% penicillin/streptomycin and 50 μM 2-mercaptoethanol.

Detection of antibodies by enzyme-linked immunosorbent assay (ELISA)

(a) Anti-dsDNA antibody ELISA. Flat-bottom 96-well plates were coated with 0.001% protamine sulfate in distilled water for 60 min, followed by 5 μg/ml of double-stranded calf

thymus DNA (Sigma-Aldrich, St. Louis, MO, USA) diluted in 0.015 M sodium citrate with 0.15 M NaCl (pH8.0) for 90 min. Plates were blocked with 1% bovine serum albumin (BSA) (Sigma-Aldrich) in PBS for 60 min. Serum samples were diluted to 1:100 with 1% BSA in PBS. Anti-dsDNA antibodies were detected with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and IgM (Bethyl Laboratories, Inc., Montgomery, TX, USA) for 60 min, followed by 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA). Reactions were terminated with 1% hydrochloric acid and then the optical density was read at 450nm.

(b) Total IgG and IgM ELISA. The levels of IgG and IgM were measured with the respective Mouse ELISA Quantitation Sets (Bethyl Laboratories), according to the manufacturer's protocols with slight modifications. Optimization of serum dilution for each test is required.

Detection of antibody-secreting cells (ASCs) by enzyme-linked immunospot (ELISpot)

To detect anti-dsDNA IgG or IgM ASCs, 96-well filter plates with 0.45 μm pore size hydrophobic PVDF membrane (Merck Millipore, Burlington, MA, USA) were pre-coated with 0.1% methylated BSA (Sigma-Aldrich), followed by 10 μg/ml DNA coating and 1% BSA in PBS as a blocking reagent. Detection of total IgG or IgM was performed by coating a plate with 15 μg/ml goat anti-mouse IgG or IgM (Bethyl Laboratories). Spots were revealed with HRP-conjugated goat anti-mouse IgG or IgM (Bethyl Laboratories), following 3-amino-9-ethylcarbazole (AEC) treatment.

Detection of blood urea nitrogen

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Blood urea nitrogen level was determined using a Urea N B kit from Wako Pure Chemical Industries (Osaka, Japan), according to the manufacturer's protocol with slight modifications. The volumes of reagents used were downsized according to the maximum volume of serum sample available.

Qualitative analysis of anti-nuclear antibodies (ANAs)

Human epithelial type 2 (HEp-2) cell substrate slides from a Fluoro HEPANA test kit (MBL, Nagoya, Japan) were used, and the procedure was based on the manufacturer's protocol with slight modifications. Serum samples were diluted to 1:40 with 1% BSA in PBS. Substrate slides were incubated with diluted serum samples for 45 min at room temperature, followed by washing three times with PBS (each time for 5 min). ANAs were detected with Alexa Fluor 488 goat anti-mouse IgG (Life Technologies, Carlsbad, CA, USA). Staining patterns were visualized and images were captured with a fluorescence microscope (BZ-9000 BioRevo from Keyence, Osaka, Japan).

Histological study

Kidneys were fixed with 4% paraformaldehyde in phosphate buffer. Paraffin-embedded samples were sectioned at 2 μm, and then stained with hematoxylin and eosin (HE) or periodic acid-Schiff (PAS). By using ImageJ software (W. Rasband, National Institutes of Health, Bethesda, MD, USA), a grid was superimposed on a kidney section captured at 400 × magnification. The total area of the glomerulus was determined by counting intersecting points on the grid in the glomerular area within Bowman's capsule. The percentage of PAS positive deposition was calculated with the formula below:

$$\% \text{ of PAS positive} = \frac{\text{no. of points falling on PAS-positive area}}{\text{total no. of points falling on glomerular area}} \times 100\%$$

At least 25 glomeruli were examined to calculate the average percentage of PAS-positive deposition.

Statistical analysis

Statistical analyses were carried out using GraphPad Prism® 6 (Version 6.0; GraphPad Software, San Diego, CA, USA) based on results of at least three independent experiments. Data are represented when appropriate as means ± SEM. Data were compared for statistical differences by one-way ANOVA with Dunn's multiple comparisons test, Pearson's correlation coefficients with two-tailed test, two-way ANOVA with Sidak's multiple comparisons test, two-tailed unpaired Student's *t*-test with Welch's correction or two-tailed paired or ratio paired Student's *t*-test as stated in the figure legends. We considered $p < 0.05$ as being statistically significant.

Results and Discussion

gp49B is expressed on plasma cells from autoimmune-prone mice

The expression of gp49B on B-lineage cells has been reported on marginal zone B (MZB) cells and memory B cells from wild type B6 mice (22). To examine the expression of gp49B on B cell subsets, particularly on plasma cells from SLE model mice, flow cytometry analysis was performed on spleen and bone marrow cells from wild type B6 mice and SLE model

BXSB/*Yaa* mice. Gp49 was expressed on MZB cells of B6 mice as reported, and also on those of BXSB/*Yaa* mice (Fig. 1A). Interestingly, plasma cells from spleens and bone marrow of BXSB/*Yaa* mice were found to exhibit robust expression of gp49, compared to in control BXSB female mice and B6 mice (Fig. 1B). High surface expression of gp49 was also noted on splenic plasma cells from other SLE mouse strains such as FcγRIIB-deficient *SLAM*^{129}, MRL/*lpr* and (NZB × NZW) F_1 mice as well as type I diabetes model NOD mice (Fig. 1B). These results coincide with our previous finding for SLE patients, i.e., plasmablasts/plasma cells from peripheral blood of the patients exhibited high expression of LILRB4 (the human counterpart of gp49B) compared to healthy controls (27). We confirmed that gp49B, but not the ITIM-less isoform gp49A, is the major subtype expressed on plasma cells by flow cytometry analysis of gp49B-deficient BXSB/*Yaa* and FcγRIIB-deficient *SLAM*^{129} mice (Fig. 1C).

*Relation of gp49B expression to the anti-dsDNA IgG serum level in BXSB/*Yaa *and Fc*γ*RIIB*$^{-/-}$SLAM^{129} mice*

We have previously shown that the LILRB4 expression level on plasmablasts/plasma cells was reduced in SLE patients after medical treatment (27). Therefore, we then checked if murine gp49B is correlated to disease development in lupus-prone mice, by performing correlation studies. We noted a significant correlation between the expression level of gp49B on splenic plasma cells and the serum titer of anti-dsDNA antibodies in BXSB/*Yaa* mice, and this was more evident in the IgG class than the IgM class (Fig. 2A). It was also observed that the gp49B level gradually increased along with disease progression and reached its peak at around 15–20 weeks old of age in BXSB/*Yaa* mice and then decreased as the mice aged (Fig. 2B).

To detect any influence of gp49B reduction on the serum autoantibody level, we then established a gp49B knockout model in the BXSB/*Yaa* and FcγRIIB$^{-/-}$*SLAM*^{129} backgrounds. Unexpectedly, gp49B genetic deletion in these model mice did not abolish serum anti-dsDNA autoantibodies. Gp49B$^{-/-}$BXSB/*Yaa* mice, however, exhibited a significant decrease in serum anti-dsDNA IgG but not the IgM class autoantibody when compared to gp49B$^{+/+}$BXSB/*Yaa* mice (Fig. 2C). On the other hand, FcγRIIB$^{-/-}$*SLAM*^{129} female mice showed a later increase of the anti-dsDNA IgG/IgM autoantibody level in serum than BXSB/*Yaa* mice. Gp49B$^{-/-}$FcγRIIB$^{-/-}$*SLAM*^{129} female mice exhibited a significantly lower, but not diminished, level of anti-dsDNA IgG at around 36 weeks old and a comparable level of the IgM class autoantibody at all ages (Fig. 2D). To determine the knockout effect on antibody-secreting cells, anti-dsDNA antibody-secreting plasma cells from spleens were detected by ELISpot assay. While gp49B deletion in BXSB/*Yaa* mice significantly decreased the total number of anti-dsDNA IgG-secreting cells, the frequencies in spleens were grossly comparable to gp49B-sufficient BXSB/*Yaa* mice (Fig. 2E). Gp49B deficiency in FcγRIIB$^{-/-}$*SLAM*^{129} mice did not cause a significant difference in the total number or frequency of pathogenic plasma cells (Fig. 2F). Examination of HEp-2-cell staining profiles of anti-nuclear autoantibodies (ANAs) for serum samples from gp49B$^{-/-}$BXSB/*Yaa* mice revealed no significant alterations in them (Supplementary Fig. S1). These results indicate that the gp49B expression level is positively correlated to the serum anti-dsDNA IgG level in both the BXSB/*Yaa* and FcγRIIB$^{-/-}$*SLAM*^{129} strains, while genetic deletion of gp49B did not abolish the autoantibody IgG, but reduced the anti-dsDNA IgG serum level due partly to the reduction in the number of the antibody-secreting cells in spleens.

*Partial amelioration of autoimmunity in gp49B$^{-/-}$ BXSB/*Yaa *mice*

We then wanted to elucidate the pathology of gp49B-deleted BXSB/*Yaa* mice in order to determine how much influence on the autoimmune disease the gp49B genetic deletion had. The gp49B$^{-/-}$BXSB/*Yaa* mice showed improved survival rate (Fig. 3A). To check the pathology of glomerulonephritis, we stained kidney sections with HE and PAS, and found that the gp49B-deficient BXSB/*Yaa* mice exhibited less severe glomerulonephritis, as indicated by tendency of the reduction of the glomerular PAS-positive deposits (Fig. 3B and 3C). We also checked blood urea nitrogen (BUN) in serum in the gp49B-deficient BXSB/*Yaa* mice, but found no significant changes or correlations to the mildly ameliorated glomerulonephritis (Supplementary Fig. S1B).

To detect any changes in cell populations other than that of plasma cells of gp49B-deficient BXSB/*Yaa* mice, we measured the splenic T and B cell as well as monocyte-macrophage populations (Fig. 4). We found a significant increase of the CD19^{+} B cells but not T cells (Fig. 4A). BXSB/*Yaa* mice are known to have depleted marginal zone B (MZB) cells (29), and an increase in peripheral monocytes (30). Interestingly, we found that gp49B$^{-/-}$BXSB/*Yaa* mice regained the MZB cell population in the spleen as well as splenic follicular B (FoB) cells (Fig. 4B). The CD21 expression level on FoB cells was also increased in gp49B$^{-/-}$BXSB/*Yaa* mice (Fig. 4C). This observation was similar to that for BXSB mice without the *Yaa* mutation, in which the intensity of CD21 on FoB cells was much lower in BXSB/*Yaa* mice (29). On the other hand, we observed a slight, but not significant, decrease in splenic monocytes from gp49B-deficient BXSB/*Yaa* mice (Fig. 4D), which might be related to the ameliorated kidney inflammation.

Collectively, we found that gp49B is expressed on plasma cells from lupus-prone, but not healthy mice. The expression level of gp49B was correlated with the serum titer of anti-dsDNA autoantibodies. However, genetic deletion of gp49B did not result in abrogation of the autoantibodies, but a slight decrease in the serum autoantibody IgG level. We also noted a modest improvement in survival rate with mild amelioration of glomerulonephritis. These results indicate that gp49B is not the sole regulator of lupus but a pathogenic element in the disease.

Defective in inhibitory receptors or a deficiency of them has been associated with the development of autoimmune disease in mice (1, 8). The presence of inhibitory receptors, for instance FcγRIIb, on plasma cells regulates their survivability and persistency in the bone marrow niche, thereby controlling antibody production (31). This could explain why plasmablasts or plasma cells from SLE-prone mice or patients exhibit reduced expression of FcγRIIb, to avoid apoptosis through FcγRIIb cross-linking (31). In contrast, we discovered up-regulation of gp49B on plasma cells from SLE-prone mice (BXSB/*Yaa*, MRL/*lpr*, NZBW F_1, $RIIB^{-/-}SLAM^{129}$) in this study, which coincides with augmented expression of human LILRB4 on plasmablasts/plasma cells from acute-phase SLE patients (27). We hypothesized that gp49B plays the opposite role from FcγRIIb, where gp49B inhibits apoptosis of plasma cells, which should be examined in the next step by referring signaling events in B-lineage cells including plasma cells from SLE-prone mice upon stimulation.

In BXSB/*Yaa* mice, we observed a relatively higher expression of gp49B on the plasma cells around 15–20 weeks old, and its expression reduced thereafter. This change of level is also reflected on the serum titer of IL-6 in BXSB/*Yaa* as reported (32), in which the level of IL-6 is peaked around 3–4.5-month-old BXSB/*Yaa* mice. It is correlated with changes of disease

manifestations such as serum titer of ANA and anti-dsDNA antibodies (32). This implies that the surface expression of gp49B could indicate the severity of disease in BXSB/*Yaa* mice. Indeed, when we deleted gp49B gene in BXSB/*Yaa*, we observed a reduction of anti-dsDNA IgG serum titer and its antibody-producing cells in the spleen. BXSB/*Yaa* mice have a 50% of mortality rate at 6 months of age, and the major cause of death is the development of immune complex-mediate glomerulonephritis (33). A milder glomerulonephritis observed in the gp49B-deficient BXSB/*Yaa* mice could contribute to the improved survival in these mice.

Although we did not demonstrate how gp49B has the direct effect on development of glomerulonephritis in BXSB/*Yaa* mice, based on our finding on the reduction of anti-dsDNA IgG titer, this might lead to slight amelioration of lupus nephritis. Presence of anti-dsDNA antibodies could contribute to the pathogenesis of glomerulonephritis as lupus nephritis can be induced in healthy mice by introducing pathogenic autoantibodies from lupus-prone mice (34), or incorporating transgene encoded anti-dsDNA IgG into normal mice (35).

Differ from BXSB/*Yaa* mice, symptoms lead to fatality in FcγRIIB$^{-/-}$*SLAM*^{129} female mice start around 8- till 12-month old (36). Deficiency of gp49B in FcγRIIB$^{-/-}$*SLAM*^{129} female mice reduced the serum titer of anti-dsDNA IgG significantly only at around 36-week old that marks the onset of autoimmunity, but not so profoundly at the later ages. The comparable titers of anti-dsDNA IgG between gp49B-sufficient and deficient FcγRIIB$^{-/-}$*SLAM*^{129} mice are also reflected from the detection of pathogenic antibody-secreting cells (Fig. 2F), because mice used for the later detection were aged between 40- and 48-week old. Difference of effects of gp49B deletion noted between BXSB/*Yaa* and FcγRIIB$^{-/-}$*SLAM*^{129} mice showed that variations still exist, despite both lupus-prone mice have commonalities to show a reduced titer of autoantibody. One possibility could be from the effect of FcγRIIB defect.

Although BXSB/*Yaa* mice and other SLE-prone mouse strains shared a common autoimmune *Fcgr2* promoter haplotype (37), complete deletion of FcγRIIB may impact differently. From this aspect, it will be appealing to understand the possible interaction between these two inhibitory receptors in generation of pathogenic plasma cells.

Integrin $\alpha_v\beta_3$ is reported to be the physiological ligand of gp49B (22, 38). Because the expression of integrin $\alpha_v\beta_3$ is found on various cell types (39), the ligation to integrin $\alpha_v\beta_3$ is controlled by its avidity state, which is upregulated only upon cellular activation (38, 40). For instance, integrin $\alpha_v\beta_3$ may ligate upon activated macrophages in response to pro-inflammatory stimuli, which will cause a sustained inflammatory response (41). Macrophages also utilize integrin $\alpha_v\beta_3$ to remove apoptotic cells (42, 43). In the case of SLE, monocytes/macrophages recognize the phagocytosed cells as an inflammatory signal and present them as autoantigens to autoreactive B and T cells, which in turn initiate autoimmunity (44). These aspects of events, particularly focusing on the ligand–gp49B interaction in macrophages and plasma cells are potential targets for further analysis.

One of the cellular abnormalities observed in BXSB/*Yaa* is the defective formation of marginal zone B (MZB) cells in the spleen. This defect is linked to the presence of the *Yaa* mutation in the B cells, regardless of the background, lupus-prone BXSB or wild-type C57BL/6 (29). It is suggested that the enhanced activation of *Yaa*-mutated MZB cells through the T-independent immune response mediated by dendritic cells prevents the accumulation of MZB cells (45). In our study, we observed an increase or recovery of the MZB cell population only in gp49B-deficient BXSB/*Yaa* mice, i.e., not in female BXSB mice (Fig. 4B and Supplementary Fig. S2A), implying the involvement of gp49B in the development of *Yaa*-bearing B cells into MZB cells. In Aiolos-deficient mice, follicular B

(FoB) cells exhibited enhanced maturation and reduction of CD21 expression, accompanied by a blockade of MZB cell formation. This is due to the elevated BCR or Btk activation resulting from the loss of Aiolos as the negative regulator (46). Aiolos-mutant mice were also reported to develop an SLE-like phenotype (47). We found that gp49 is expressed on FoB cells from BXSB mice with the *Yaa* mutation (Supplementary Fig. S2B), different from in the case of the C57BL/6 background reported (22), which suggests that gp49B possibly negatively regulates the Aiolos pathway, and thus enhances BCR or Btk activation in the *Yaa* background. This could explain the observation of the higher CD21 level on FoB cells from gp49B$^{-/-}$BXSB mice with the *Yaa* mutation (Fig. 4C, and Supplementary Fig. S2C), and MZB cell recovery. The *Yaa* mutation with MZB cell loss is not associated with the development of lupus in BXSB mice. This is seen in BXSB.H-2^{d} and BXSB.Eα mice that have a defect in the MZB cell compartment, but are protected from lupus manifestation (29, 48, 49). Thus, even though MZB cells were regained in the gp49B-deficient BXSB/*Yaa* mice, this may not contribute significantly to the reduction of autoantibody IgG production indicated in Fig. 2C.

In conclusion, although we failed to determine the exact mechanism for the underlying relationship between gp49B and disease progression, gp49B could be a marker for pathogenic plasma cells of lupus-prone mice. Depletion of plasma cells as well as other gp49B-positive pathogenic cells will most likely clarify the exact role of this inhibitory receptor in autoimmunity.

Declaration of COI

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TT collaborated with Ono Pharmaceutical Industry Ltd in this study. Other authors declare no conflict of interest.

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References

1. Olde Nordkamp, M.J.M., Koeleman, B.P., and Meyaard, L. 2014. Do inhibitory immune receptors play a role in the etiology of autoimmune disease? *Clin. Immunol.* 1:31.
2. Daëron, M., Jaeger, S., Du Pasquier, L., and Vivier, E. 2008. Immunoreceptor tyrosine-based inhibition motifs: a quest in the past and future. *Immunol. Rev.* 1:11.
3. Parry, R.V., Chemnitz, J.M., Frauwirth, K.A.*, et al.* 2005. CTLA-4 and PD-1 Receptors Inhibit T-Cell Activation by Distinct Mechanisms. *Mol. Cell. Biol.* 21:9543.
4. Fife, B.T. and Bluestone, J.A. 2008. Control of peripheral T-cell tolerance and autoimmunity via the CTLA-4 and PD-1 pathways. *Immunol. Rev.* 1:166.

5. Ansari, M.J.I., Salama, A.D., Chitnis, T.*, et al.* 2003. The Programmed Death-1 (PD-1) Pathway Regulates Autoimmune Diabetes in Nonobese Diabetic (NOD) Mice. *J. Exp. Med.* 1:63.

6. Morris, G.P., Brown, N.K., and Kong, Y.-c.M. 2009. Naturally-existing CD4+CD25+Foxp3+ regulatory T cells are required for tolerance to experimental autoimmune thyroiditis induced by either exogenous or endogenous autoantigen. *J. Autoimmun.* 1:68.

7. Kong, Y.-c.M. and Flynn, J.C. 2014. Opportunistic Autoimmune Disorders Potentiated by Immune-Checkpoint Inhibitors Anti-CTLA-4 and Anti-PD-1. *Front Immunol*. 206.

8. Pritchard, N.R. and Smith, K.G.C. 2003. B cell inhibitory receptors and autoimmunity. *Immunology*. 3:263.

9. Pritchard, N.R., Cutler, A.J., Uribe, S., Chadban, S.J., Morley, B.J., and Smith, K.G.C. 2000. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcγRII. *Curr. Biol.* 4:227.

10. Kyogoku, C., Dijstelbloem, H.M., Tsuchiya, N.*, et al.* 2002. Fcγ receptor gene polymorphisms in Japanese patients with systemic lupus erythematosus: Contribution of FCGR2B to genetic susceptibility. *Arthritis Rheum.* 5:1242.

11. Chu, Z.T., Tsuchiya, N., Kyogoku, C.*, et al.* 2004. Association of Fcγ receptor IIb polymorphism with susceptibility to systemic lupus erythematosus in Chinese: a common susceptibility gene in the Asian populations. *Tissue Antigens*. 1:21.

12. Kanari, Y., Sugahara-Tobinai, A., Takahashi, H.*, et al.* 2014. Dichotomy in FcγRIIB deficiency and autoimmune-prone SLAM haplotype clarifies the roles of the Fc receptor in development of autoantibodies and glomerulonephritis. *BMC Immunol.*:47.

13. Arm, J.P., Gurish, M.F., Reynolds, D.S.*, et al.* 1991. Molecular cloning of gp49, a

cell-surface antigen that is preferentially expressed by mouse mast cell progenitors and is a new member of the immunoglobulin superfamily. *J. Biol. Chem.* 24:15966.

14. Castells, M.C., Wu, X., Arm, J.P., Austen, K.F., and Katz, H.R. 1994. Cloning of the gp49B gene of the immunoglobulin superfamily and demonstration that one of its two products is an early-expressed mast cell surface protein originally described as gp49. *J. Biol. Chem.* 11:8393.

15. Lee, K.H., Ono, M., Inui, M., Yuasa, T., and Takai, T. 2000. Stimulatory Function of gp49A, a Murine Ig-Like Receptor, in Rat Basophilic Leukemia Cells. *J. Immunol.* 9:4970.

16. Katz, H.R., Vivier, E., Castells, M.C., McCormick, M.J., Chambers, J.M., and Austen, K.F. 1996. Mouse mast cell gp49B1 contains two immunoreceptor tyrosine-based inhibition motifs and suppresses mast cell activation when coligated with the high-affinity Fc receptor for IgE. *Proc. Natl. Acad. Sci. U.S.A.* 20:10809.

17. Lu-Kuo, J.M., Joyal, D.M., Austen, K.F., and Katz, H.R. 1999. gp49B1 inhibits IgE-initiated mast cell activation through both immunoreceptor tyrosine-based inhibitory motifs, recruitment of src homology 2 domain-containing phosphatase-1, and suppression of early and late calcium mobilization. *J. Biol. Chem.* 9:5791.

18. Zhou, J.S., Friend, D.S., Feldweg, A.M.*, et al.* 2003. Prevention of lipopolysaccharide-induced microangiopathy by gp49B1: evidence for an important role for gp49B1 expression on neutrophils. *J. Exp. Med.* 8:1243.

19. Daheshia, M., Friend, D.S., Grusby, M.J., Austen, K.F., and Katz, H.R. 2001. Increased severity of local and systemic anaphylactic reactions in gp49B1-deficient mice. *J. Exp. Med.* 2:227.

20. Kasai, S., Inui, M., Nakamura, K.*, et al.* 2008. A novel regulatory role of gp49B on dendritic cells in T-cell priming. *Eur. J. Immunol.* 9:2426.

21. Wang, L.L., Chu, D.T., Dokun, A.O., and Yokoyama, W.M. 2000. Inducible expression of the gp49B inhibitory receptor on NK cells. *J. Immunol.* 10:5215.

22. Fukao, S., Haniuda, K., Nojima, T., Takai, T., and Kitamura, D. 2014. gp49B-mediated negative regulation of antibody production by memory and marginal zone B cells. *J Immunol*. 2:635.

23. Kang, X., Kim, J., Deng, M.*, et al.* 2016. Inhibitory leukocyte immunoglobulin-like receptors: Immune checkpoint proteins and tumor sustaining factors. *Cell Cycle*. 1:25.

24. Arm, J.P., Nwankwo, C., and Austen, K.F. 1997. Molecular identification of a novel family of human Ig superfamily members that possess immunoreceptor tyrosine-based inhibition motifs and homology to the mouse gp49B1 inhibitory receptor. *J. Immunol.* 5:2342.

25. Thomas, R., Matthias, T., and Witte, T. 2010. Leukocyte Immunoglobulin-Like Receptors as New Players in Autoimmunity. *Clin Rev Allergy Immunol*. 2:159.

26. Inui, M., Hirota, S., Hirano, K.*, et al.* 2015. Human CD43+ B cells are closely related not only to memory B cells phenotypically but also to plasmablasts developmentally in healthy individuals. *Int. Immunol.* 7:345.

27. Inui, M., Sugahara-Tobinai, A., Fujii, H.*, et al.* 2016. Tolerogenic immunoreceptor ILT3/LILRB4 paradoxically marks pathogenic auto-antibody-producing plasmablasts and plasma cells in non-treated SLE. *Int. Immunol.* 12:597.

28. Takai, T., Ono, M., Hikida, M., Ohmori, H., and Ravetch, J.V. 1996. Augmented humoral and anaphylactic responses in FcγRII-deficient mice. *Nature*.346.

29. Amano, H., Amano, E., Moll, T.*, et al.* 2003. The *Yaa* Mutation Promoting Murine Lupus Causes Defective Development of Marginal Zone B Cells. *J. Immunol.* 5:2293.

30. Wofsy, D., Kerger, C.E., and Seaman, W.E. 1984. Monocytosis in the BXSB model for systemic lupus erythematosus. *J. Exp. Med.* 2:629.

31. Xiang, Z., Cutler, A.J., Brownlie, R.J.*, et al.* 2007. FcγRIIb controls bone marrow plasma cell persistence and apoptosis. *Nat. Immunol.*:419.

32. Jain, S., Park, G., Sproule, T.J., *et al.* 2016. Interleukin 6 Accelerates Mortality by Promoting the Progression of the Systemic Lupus Erythematosus-Like Disease of BXSB.Yaa Mice. *PLoS One* 11: e0153059.

33. Theofilopoulos, A.N., McConahey, P.J., Izui, S., Eisenberg, R.A., Pereira, A.B. and Creighton, W.D. 1980. A comparative immunologic analysis of several murine strains with autoimmune manifestations. *Clin. Immunol. Immunopathol.* 15:258.

34. Vlahakos, D.V., Foster, M.H., Adams, S., *et al.* 1992. Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites. *Kidney Int.* 41:1690.

35. Tsao, B.P., Ohnishi, K., Cheroutre, H., *et al.* 1992. Failed self-tolerance and autoimmunity in IgG anti-DNA transgenic mice. *J. Immunol.* 149:350.

36. Boross, P., Arandhara, V.L., Martin-Ramirez, J., *et al.* 2011. The inhibiting Fc receptor for IgG, FcγRIIB, is a modifier of autoimmune susceptibility. *J. Immunol.* 187:1304.

37. Pritchard, N.R., Cutler, A.J., Uribe, S., Chadban, S.J., Morley, B.J. and Smith, K.G. 2000. Autoimmune-prone mice share a promoter haplotype associated with reduced expression and function of the Fc receptor FcgammaRII. *Curr. Biol.* 10:227.

38. Castells, M.C., Klickstein, L.B., Hassani, K.*, et al.* 2001. gp49B1-αvβ3 interaction inhibits antigen-induced mast cell activation. *Nat. Immunol.*:436.

39. Felding-Habermann, B. and Cheresh, D.A. 1993. Vitronectin and its receptors. *Curr. Opin. Cell Biol.* 5:864.

40. van Kooyk, Y. and Figdor, C.G. 2000. Avidity regulation of integrins: the driving force in leukocyte adhesion. *Curr. Opin. Cell Biol.* 5:542.

41. Antonov, A.S., Antonova, G.N., Munn, D.H.*, et al.* 2011. αVβ3 integrin regulates

macrophage inflammatory responses via PI3 kinase/Akt-dependent NF-κB activation. *J. Cell. Physiol.* 2:469.

42. Savill, J., Dransfield, I., Hogg, N., and Haslett, C. 1990. Vitronectin receptor-mediated phagocytosis of cells undergoing apoptosis. *Nature*.170.

43. Fadok, V.A., Savill, J.S., Haslett, C.*, et al.* 1992. Different populations of macrophages use either the vitronectin receptor or the phosphatidylserine receptor to recognize and remove apoptotic cells. *J. Immunol.* 12:4029.

44. Katsiari, C.G., Liossis, S.-N.C., and Sfikakis, P.P. 2010. The Pathophysiologic Role of Monocytes and Macrophages in Systemic Lupus Erythematosus: A Reappraisal. *Semin. Arthritis Rheum.* 6:491.

45. Santiago-Raber, M.-L., Amano, H., Amano, E.*, et al.* 2010. Evidence that Yaa-induced loss of marginal zone B cells is a result of dendritic cell-mediated enhanced activation. *J. Autoimmun.* 4:349.

46. Cariappa, A., Tang, M., Parng, C.*, et al.* 2001. The Follicular versus Marginal Zone B Lymphocyte Cell Fate Decision Is Regulated by Aiolos, Btk, and CD21. *Immunity*. 5:603.

47. Sun, J., Matthias, G., Mihatsch, M.J., Georgopoulos, K., and Matthias, P. 2003. Lack of the Transcriptional Coactivator OBF-1 Prevents the Development of Systemic Lupus Erythematosus-Like Phenotypes in Aiolos Mutant Mice. *J. Immunol.* 4:1699.

48. Merino, R., Fossati, L., Lacour, M., Lemoine, R., Higaki, M., and Izui, S. 1992. H-2-linked control of the Yaa gene-induced acceleration of lupus-like autoimmune disease in BXSB mice. *Eur. J. Immunol.* 2:295.

49. Merino, R., Iwamoto, M., Fossati, L.*, et al.* 1993. Prevention of systemic lupus erythematosus in autoimmune BXSB mice by a transgene encoding I-E alpha chain. *J. Exp. Med.* 4:1189.

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Figure Legends

Figure 1. The expression level of gp49B on plasma cells.

(A) Representative flow cytometry histograms of gp49 expression on marginal zone B cells from C57BL/6 and BXSB/*Yaa* mice.

(B) Representative flow cytometry histograms of gp49 expression on the plasma cells from spleen and bone marrow from wild type mouse and SLE model mice (*top*). MFI of gp49 was normalized to that of isotype control antibody and data are represented as means ± SEM from at least three individual experiments (*bottom*). *P* values calculated by one-way ANOVA with Dunn's multiple comparisons test (C57BL/6 as control column) are represented in asterisk sign (*). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. *P* values calculated by two-tailed unpaired Student's *t*-test with Welch's correction are represented in dagger sign (†). † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$, †††† $p < 0.0001$.

(C) Representative flow cytometry histograms of gp49 expression on splenic plasma cells from gp49B$^{+/+}$ or gp49B$^{-/-}$ in BXSB/*Yaa* and B6.FcγRIIB-deficient *SLAM*^{129} (FcγRIIB$^{-/-}$*SLAM*^{129}) mice.

Figure 2. The relationship between gp49B expression and anti-dsDNA IgG serum titer.

(A) Correlation between the gp49 expression level on splenic plasma cells from BXSB/*Yaa* mice and the serum titers of anti-dsDNA IgG (*left*) and IgM (*right*). *P* values were calculated using Pearson's correlation coefficients with a two-tailed test.

(B) Correlation between the gp49 expression level on splenic plasma cells from BXSB/*Yaa* mice and the age of mice (weeks). *P* value was calculated using Pearson's correlation coefficients with a two-tailed test.

(C) Sera were collected from gp49B$^{+/+}$ and gp49B$^{-/-}$BXSB/*Yaa* mice. The levels of anti-dsDNA IgG (*left*) and IgM (*right*) were determined by ELISA. Horizontal lines represent mean values ± SEM. *P* value is calculated by two-tailed paired Student's *t*-test. * $p < 0.05$.

(D) Sera were collected from gp49B$^{+/+}$ and gp49B$^{-/-}$FcγRIIB$^{-/-}$*SLAM*^{129} at 4-week intervals from 24-weeks old to 48-weeks old. The levels of anti-dsDNA IgG (*left*) and IgM (*right*) were determined by ELISA. Horizontal lines represent mean values. *P* value was calculated by two-way ANOVA with Sidak's multiple comparisons test. * $p < 0.05$.

(E) The total number (*top*) and frequency (*bottom*) of anti-dsDNA IgG- and IgM-secreting cells among sorted splenic plasma cells of gp49B$^{+/+}$ and gp49B$^{-/-}$BXSB/*Yaa* mice were determined by ELISpot assay. Horizontal lines represent mean values ± SEM. *P* value was calculated using two-tailed paired Student's *t*-test for each group. * $p < 0.05$.

(F) The total number (*top*) and frequency (*bottom*) of anti-dsDNA IgG- and IgM-secreting cells from sorted splenic plasma cells of gp49B$^{+/+}$ and gp49B$^{-/-}$FcγRIIB$^{-/-}$*SLAM*^{129} mice were determined by ELISpot assay. Horizontal lines represent mean values ± SEM.

Figure 3. Partial amelioration of autoimmunity in gp49B$^{-/-}$BXSB/*Yaa* mice.

(A) Survival curves for BXSB/*Yaa* mice (gp49B$^{+/+}$, $n = 30$; gp49B$^{-/-}$, $n = 18$). Mice were monitored for survival until week 50. The median survival for gp49B$^{+/+}$BXSB/*Yaa* mice is until week 26, while that for gp49B$^{-/-}$BXSB/*Yaa* mice is undefined. *P* value is calculated by Log-rank (Mantel-Cox) test.

(B) Representative images of kidney sections from gp49B$^{+/+}$ and gp49B$^{-/-}$BXSB/*Yaa* and female gp49B$^{+/+}$BXSB mice stained with HE (*above*) and PAS (*below*). Original magnification, × 400. Scale bar, 100 μm.

(C) Percentage of deposition of PAS positive material on glomerular area within Bowman's capsule was calculated as depicted in *Materials and Methods*. Data are represented as means

± SEM from five individual experiments, excepting the plot of female gp49B$^{+/+}$BXSB for single determination. *P* value is calculated by two-tailed paired Student's *t*-test.

Figure 4. Changes of cell populations in gp49B$^{-/-}$BXSB/*Yaa* mice.

(A) The frequencies of CD19^{+} B cells (*left*) and CD3^{+} T cell subsets (*right*) from spleens were analyzed by flow cytometry. Horizontal lines represent mean values ± SEM. *P* value is calculated by two-tailed paired Student's *t*-test. * $p < 0.05$.

(B) Representative flow cytometric data on follicular B cells (FoB) and marginal zone B cells (MZB), gated from CD19^{+} B cells of gp49B$^{+/+}$ and gp49B$^{-/-}$BXSB/*Yaa* mice (*top*). The frequencies of FoB and MZB are represented as means ± SEM (*bottom*). *P* value is calculated by two-tailed paired Student's *t*-test. * $p < 0.05$, *** $p < 0.001$.

(C) Representative flow cytometric histogram of expression of CD21 on follicular B cells (*top*). The MFI of CD21 expression are represented as means ± SEM (*bottom*). *P* value is calculated by two-tailed ratio paired *t*-test. * $p < 0.05$.

(D) Representative flow cytometric data on spleen monocytes (B220^{-}CD11b^{+}) of gp49B$^{+/+}$ and gp49B$^{-/-}$BXSB/*Yaa* mice (*left)*. The frequencies of monocytes are represented as means ± SEM (*right*). *P* value is calculated by two-tailed paired *t*-test.