PLD4 is a genetic determinant to systemic lupus erythematosus and involved in murine autoimmune phenotypes

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ABSTRACT

Objectives Systemic lupus erythematosus (SLE) is an autoimmune disease that is characterised by autoantibody production and widespread inflammation damaging many organs. Previous genome-wide association studies (GWASs) have revealed over 80 genetic determinants of SLE, but they collectively explain a fraction of the heritability, and only a few were proven in vivo for the involvement in SLE. We conducted a meta-analysis of SLE GWAS in the Japanese population, followed by functional analyses of a susceptibility gene with use of mutant mice.

Methods We conducted a meta-analysis of two GWASs comprising a total of 1363 cases and 5536 controls using the 1000 Genome Project data as an imputation reference. Enrichment analyses for functional annotations were conducted. We examined Phospholipase D4 (Pld4) mutant mice to assess functional involvement of a genetic determinant.

Results We found a total of 14 significant loci, which included rs2582511 in AHNAK2/PLD4 recently reported in a Chinese study and a novel locus of rs143181706 in MAMLD1 (p=7.9×10−11 and 3.7×10−8, respectively). PLD4 risk allele was associated with anti-dsDNA antibody production. Enrichment analysis of genetic signals revealed involvement of a wide range of immune-related cells and pathways. Pld4 mutant mice revealed remarkably low body weight. The mice demonstrated autoimmune phenotypes compatible with SLE, including splenomegaly and lymphadenopathy, expansion of B cells and hypersecretion of BAFF and production of autoantibodies especially anti-nuclear antibody and anti-dsDNA antibody.

Conclusions We found a novel susceptibility gene to SLE. Pld4 mutant mice revealed autoimmune phenotypes suggesting functional involvement of PLD4 with the basics of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoantibody-mediated autoimmune disease characterised by systemic organ involvement. A variety of autoantibodies including anti-nuclear antibody (ANA), anti-DNA antibody, anti-Ro antibody and anti-RNP antibody frequently accompany SLE. More than 80% of the patients with SLE are young women at the onset of this disease. Severe organ damages include renal failure and central nervous system (CNS) lupus, both of which are sometimes fatal. Serologically, type I interferon, especially interferon alpha signature, is assumed to play critical roles in autoimmune diseases and lead to development of new therapeutic strategies.

Key messages

What is already known about this subject

Genetic studies, mainly genome wide association study (GWAS), for SLE so far have identified more than 80 susceptibility loci. However, a quite few of them have been shown their functional involvement in SLE in vivo.

What does this study add

We conducted a meta-analysis of GWAS for SLE in Japanese population, and identified two new susceptibility genes, PLD4 and MAMLD1. PLD4 is a susceptibility gene to RA and SSc.

PLD4 risk allele was associated with production of anti-dsDNA antibody, suggesting that PLD4 contributes to SLE pathophysiology by activating B cells.

Pld4 mutant mice demonstrated autoimmune phenotypes corresponding to SLE, including the production of anti-nuclear antibody and pathology of nephritis.

How might this impact on clinical practice or future developments

PLD4 is a causative gene shared by multiple human autoimmune diseases (RA, SSc, SLE) and seems to have a role in the general immune system beyond species.

Since PLD4 biological function has not been fully elucidated, clarification of biology of PLD4 would deepen our understanding of autoimmune diseases and lead to development of new therapeutic strategies.
Although treatments are available for this autoimmune disorder, environmental and genetic components are associated with the onset of SLE and more research is required to develop additional comprehensive therapies. A previous study analysing 104 monozygotic twins reported 24% concordance for SLE, much higher than that in rheumatoid arthritis (RA). Genetic components are estimated to explain about 44% to 66% of the onset of SLE. HLA is the strongest genetic component among the susceptibility loci. Previous genetic studies, mainly genome-wide association studies (GWASs), have identified a total of more than 80 susceptibility loci to SLE, some of which were ethnic specific. However, these loci collectively explain a mere ~24% of heritability and there are a number of yet-to-be-identified genetic loci. Furthermore, it is a challenge to narrow down a single causative gene and variant out of the candidate susceptibility loci.

Despite identifying over 80 susceptibility loci to SLE, a few genes or variants were functionally examined for their associations with SLE pathophysiology.

Expansion of subjects in a single population is an effective approach to identify susceptibility loci which is population specific or difficult to be found through trans-ethnic meta-analysis due to different linkage disequilibrium (LD) structure or allele frequencies. In fact, previous Asian-specific approach seemed to be successful in uncovering genetic determinants for SLE. While so far there have been numerous GWASs conducted in European and Asian populations, there is only one GWAS from the Japanese population in which a total of six non-HLA loci were identified, five of which were shared by Europeans. To expand our knowledge of genetic background of SLE and its functional relevance, here we conducted a meta-analysis of the previous Japanese GWAS and a de novo GWAS. In addition, mutant mouse of Pld4, a susceptibility gene to SLE, was examined to get an insight of SLE pathophysiology.
intercept before and after LD score regression to estimate polygenic effects on SLE susceptibility.

Clinical information
Presence of autoantibodies in SLE, namely, anti-DNA antibody, anti-SSA antibody, anti-SSB antibody and anti-U1RNP antibody, were obtained from clinical charts. Since all of the subjects in set 1 were derived from a single institution, clinical information was intensively analysed in set 1 to avoid data noises arising from different measurement methods.

Functional annotation and enrichment
Variants with significant associations were functionally annotated with the use of Haploreg16 to evaluate enrichment of enhancer histone marks in significant variants or variants in LD with them.

LD estimation
LD between variants were estimated by plink software.17

Heritability estimate and partitioning heritability
Heritability explained by a single variant or a set of significant variants was calculated by liability threshold model assuming prevalence of SLE as 0.05%,1 the details of which are described elsewhere.18 Partitioning of heritability into cell groups or detailed cell types was conducted by ldsc software.15

Pathway analysis
Pathway analysis for the evaluation of important molecular networks based on genetic association results was conducted by using PASCAL software.19 We adopted the method to calculate gene scores by taking sum of all variants, not restricted to top variants, in gene regions into account.
Statistical analysis for genetic studies

Logistic regression analysis was performed for this study, and the covariates for each GWAS are shown in online supplementary table 2. Statistical analysis was conducted by plink1.9 software or mach2dat software for sets 1 and 2, respectively. Meta-analysis was conducted with the use of the inverse-variance method assuming fixed effects by R statistical software. As for the X chromosome, we separately conducted association studies for men and women and combined the results in each set. Significant level was set as $5.0 \times 10^{-8}$ for GWAS and 0.05 for clinical phenotypes and mice study. To confirm a significant association between a clinical phenotype and genotype, we further conducted a permutation test with 10,000 permutations by shuffling genotypes to take covariance structure among auto-antibody positivity into account and obtained null distribution of the smallest p values.

In vivo study of Pld4

Mice and cells

BALB/c Pld4 thss/thss mutants were purchased from The Jackson Laboratory (JAX stock #012624) (BH, Submission, 2011). All animal studies used three to six female mice that are 12–15 weeks of age, repeated at least two times, unless otherwise indicated. All mice were maintained in a specific pathogen-free condition at Kyoto University, Japan. All experiments were approved by the Animal Care and Use Committee of the Institute for Kyoto University and were performed in accordance with the institutional guidelines. For cell culture experiment, splenic CD19⁺ cells were isolated using mouse CD19 microbeads combined with MACS Column according to the manufacturer’s protocol (Miltenyi Biotec). The purity of CD19⁺ cells was typically 85%–90%.

Confirmation of genotypes

DNA was extracted from tail or ear of mice and subjected to either Sanger sequencing or PCR followed by restriction digest by HpyCH4III enzyme. Details are described in online Supplementary Methods.

Analysis of body weight and organs

Body weight was measured every week from birth for both mutant and heterozygous mice. Weights of fresh organs were compared between the two genotypes at 1 month after birth.

Antibodies, cell culture and flow cytometric analysis

Monoclonal antibodies used for the current study are described in online Supplementary Methods. For B-cell proliferation assay, the purified CD19⁺ cells were labelled with 5 µM carboxyfluorescein succinimidyl ester (CFSE) for 5 min at room temperature, then washed three times with phosphate buffered saline (PBS) containing 5% fetal calf serum (FCS) and cultured at a density of $1 \times 10^5$ cells/mL in RPMI 1640 supplemented with 10% FCS, 50 mM β-mercaptoethanol, 2 mM glutamine, 100 U/mL penicillin and 100 mg/mL streptomycin for 72 hours, in the presence or absence of goat anti-mouse IgM (Southern Biotech), lipopolysaccharide (LPS) (Sigma-Aldrich) and CpG oligodeoxynucleotides (CpG ODN) 2395 (Miltenyi Biotec) at the indicated concentrations. The dividing cells were determined as the cells with reduced intensity of CFSE labelling measured by flow cytometry. All stained cells were analysed by FACScalibur (BD Biosciences).

Histology and immunohistochemistry

For histology, every organ was fixed in 10% formalin, embedded in paraffin, and the sections were stained with hematoxylin and eosin (H&E) and Periodic acid-Schiff (PAS) stain. For immunohistochemistry of the kidney, the organs were snap frozen in isopentane with dry ice, mounted in optimal cutting temperature (OCT) compound and stored at −80°C. Cryostat sections at 10 µm were cut and mounted on gelatin-coated histological slides. Sections were air dried and fixed with ice-cold acetone for 20 min. After blocking for non-specific staining with PBS containing 2% FCS, sections were incubated with FITC-conjugated anti-mouse IgG antibody for 60 min at 4°C and rinsed with PBS. For immunohistochemistry of the spleen, snap-frozen sections were fixed with ice-cold acetone for 5 min, washed with PBS, blocked with Blocking One (Nacalai Tesque, Japan) for 30 min and then incubated with indicated antibodies for 60 min or streptavidin for 30 min at room temperature. All images were obtained by fluorescence microscope FSX100 (Olympus). All slides were prepared by the Center for Anatomical, Pathological and Forensic Medical Research, Kyoto University Graduate School of Medicine.

ELISA and cytometric bead array (CBA) assay

We quantified serum levels of BAFF by Mouse BAFF/Blys Quantikine ELISA kit according to the manufacturer’s protocol (R&D Systems, USA). For determining concentration of serum immunoglobulin in mice, we used Mouse Immunoglobulin Isotyping Kit according to the manufacturer’s protocol (BD Biosciences).

ANA and anti-dsDNA antibody detection

We determined serum ANA by using ImmuGlo ANA Hep-2 substrate according to the manufacturer’s protocol. For detection of anti-nuclear antibody towards mouse antigen, $5 \times 10^5$ mouse embryonic fibroblast cells were plated on 24 flat-bottom well, culture dish and cultured overnight. Attached cells were fixed with 0.5% formaldehyde for 5 min, then permeated with 0.1% Triton-X. After washing the cells, serially diluted sera were applied, followed by FITC-conjugated anti-mouse IgG secondary antibody. Titre of ANA was defined as the serum dilution at which fluorescence was no longer visible. For anti-dsDNA antibody, Mouse Anti-dsDNA ELISA Kit (Shibayagi, Japan) was used following the manufacturer’s instructions.

Gene expression analysis

We extracted total RNA from kidney and splenocyte using RNaseasy mini kit (QIAGEN, Germany). A total of 1 µg of the RNA was reverse-transcribed into cDNA with High Capacity cDNA Reverse Transcription Kits (Applied Biosystems, USA). For the gene expression analysis of kidney, control glyceraldehyde-3-phosphate dehydrogenase and target cDNAs were amplified using SYBR green method. Details of primers are described in online Supplementary Methods. For splenocytes, mRNA expression of IL-6, IL-12p, TNF-α and IL-1b were obtained using TaqMan Gene Expression Assay (Applied Biosystems), according to the manufacturer’s protocol. We conducted real-time PCRs with the use of the ABI 7500 system (Applied Biosystems).

Statistical analysis for mice studies

SPSS software was used for analyses (IBM, USA). Welch’s t-test or Fisher’s exact test was carried out for statistical analysis using Prism V6 software (GraphPad, USA). P values less than 0.05 were considered significant.
were identified (rs2582511, \( p = 7.9 \times 10^{-7} \) and \( p \leq 1.0 \times 10^{-6} \)). BMP2K and two loci were previously reported for the remaining 12 loci. We found no independent signals (\( p > 0.08 \)). Association with SLE susceptibility SNPs showed comparable effect sizes across the two studies (Table 1 and figure 1B). We further analysed whether rs2582511 and rs143181706 were associated with clinical phenotypes of SLE. We found that rs2582511 risk allele was associated with increased positivity of anti-dsDNA antibody (nominal \( p = 0.0073 \), Table 2). We also

### Results

A total of 1363 cases and 5536 controls in two GWASs were imputed for genome-wide SNPs using 1000 G p3v5 panel as reference. We conducted logistic regression analysis and combined the results by inverse-variance method to assess the overall significance.

As a result, there was no evidence for confounding bias leading to deviation of \( \chi^2 \) statistics from expectation across our data set (lambda = 1.08, figure 1A). The HLA region showed the strongest signal (rs796780915, \( p = 1.08 \times 10^{-11} \)) and MAMLD1 on chromosome X, a novel susceptibility locus, was identified (rs2582511, \( p = 7.9 \times 10^{-11} \) and rs143181706, \( p = 3.7 \times 10^{-10} \), respectively, Table 1 and figure 1B). Both susceptibility SNPs showed comparable effect sizes across the two studies (Table 1). Conditioning on the top SNP in each region, we did not identify independent signals (\( p > 0.08 \)). Association with SLE was previously reported for the remaining 12 loci. We found two loci (BMP2K and PTGER4) showing suggestive associations (\( p \leq 1.0 \times 10^{-5} \)) which were not reported previously (Table 1).

We further found evidence of genetic similarities between the current and previous studies. Our results contained 39 top SNPs previously reported as SLE susceptibility loci and not in Table 1 (we excluded susceptibility loci from Asian studies containing part of our results). Twenty-six of the 39 markers showed \( p \) values less than 0.05 and 37 out of the 39 shared risk alleles (online supplementary table 3), indicating strong genetic overlap across populations.

rs2582511 is a synonymous variant of AHNK2 which encodes a nucleoprotein involved with calcium signalling and its role in carcinogenesis has been previously suggested. The LD block containing rs2582511 spanned 400 kb and contained AHNK2 and PLD4 which encodes a member of the phospholipase family without phospholipase D activity. In an intronic region of MAMLD1, MAMLD1 is the only gene in the LD block containing rs143181706 (figure 2B) and is associated with genital development including hypospadias and gonadal dysgenesis.

### Table 1  Genetic associations with SLE susceptibility found in the current study

| SNP | Chr | position | Gene | Ref | Alt | Alt freq | Case/Cont | \( P \) value | Alt freq | Case/Cont | \( P \) value | Meta-analysis |
|-----|-----|----------|------|-----|-----|----------|------------|-----------|----------|----------|------------|---------------|---------------|
| Significant loci | 1 | 173191371 | TNFSF4 | A | G | 0.36/0.29 | 5.1 \times 10^{-6} | 0.34/0.29 | 1.3 \times 10^{-7} | 3.4 \times 10^{-12} | 1.39 (1.27 to 1.52) |
| | 2 | 191943742 | STAT4 | C | T | 0.40/0.3 | 1.3 \times 10^{-4} | 0.39/0.29 | 6.4 \times 10^{-7} | 2.9 \times 10^{-24} | 1.59 (1.45 to 1.73) |
| | 14 | 105416010 | AHNAK2/PLD4 | T | C | 0.30/0.38 | 2.1 \times 10^{-6} | 0.33/0.38 | 5.6 \times 10^{-6} | 7.9 \times 10^{-11} | 1.32 (1.2 to 1.48) |
| | 3 | 11889341 | TNFAIP3 | C | T | 0.12/0.07 | 4.6 \times 10^{-7} | 0.11/0.07 | 6.1 \times 10^{-8} | 6.0 \times 10^{-14} | 1.43 (1.3 to 1.56) |
| | 10 | 5090077 | PCDH15, WDFY4 | G | T | 0.20/0.15 | 0.00036 | 0.20/0.15 | 0.0034 | 2.0 \times 10^{-8} | 1.94 (1.83 to 2.06) |
| | 14 | 105416010 | AHNAK2/PLD4 | T | C | 0.30/0.38 | 2.1 \times 10^{-6} | 0.33/0.38 | 5.6 \times 10^{-6} | 7.9 \times 10^{-11} | 1.32 (1.2 to 1.48) |
| | 11 | 128304141 | ETS1 | C | A | 0.46/0.04 | 0.00036 | 0.48/0.39 | 8.7 \times 10^{-13} | 1.8 \times 10^{-13} | 1.34 (1.25 to 1.43) |
| | 5 | 106565056 | PRDM1 | G | A | 0.46/0.3 | 0.011 | 0.48/0.34 | 2.6 \times 10^{-5} | 8.6 \times 10^{-7} | 1.25 (1.14 to 1.36) |

### Table 2  Significant association between rs2582511 and anti-dsDNA antibody production in intracase analyses

| Phenotype | Number of positive subjects* (%) | \( OR \) (95% CI) | \( P \) value | \( OR \) (95% CI) | \( P \) value |
|-----------|---------------------------------|-----------------|------------|-----------------|------------|
| Anti-dsDNA | 245 (59.3) | 1.52 (1.12 to 2.06) | 0.0073 | 1.05 (0.71 to 1.54) | 0.81 |
| Anti-RNP   | 245 (57.6) | 1.23 (0.92 to 1.66) | 0.16 | 1.12 (0.77 to 1.63) | 0.56 |
| Anti-SSA   | 280 (65.1) | 1.11 (0.82 to 1.51) | 0.49 | 1.07 (0.72 to 1.57) | 0.74 |
| Anti-SSB   | 106 (32.0) | 1.32 (0.92 to 1.9) | 0.14 | 1.13 (0.72 to 1.78) | 0.59 |

*Subjects positive for antibodies in set 1. Subjects with information of the autoantibodies set as a denominator.
10% of risk allele for systemic lupus erythematosus.

**Table 1** Genetic associations with SLE susceptibility found in the current study

**Table 2** Significant association between rs2582511 and anti-dsDNA antibody production in intracase analyses
found that the risk alleles in the two SNPs showed positive associations (OR > 1) with all of the autoantibodies analysed (permutation p=0.006, table 2), suggesting PLD4 works on B-cell hyperactivity.

When we calculated heritability explained by the two SNPs based on a liability-scale threshold model, rs2582511 and rs143181706 explained 0.37% and 0.26% of heritability, respectively. A total of 5.7% of heritability was brought about by the 14 significant SNPs in the current study.

We found that significant non-HLA SNPs and their linked SNPs showed enrichment of enhancer histone marks in disease-relevant cell types including B cells and NK cells by Haploreg (online supplementary table 4).

In order to assess polygenic effects on SLE susceptibility, we conducted LD score regression. Since we found a strong correlation between LD scores and χ² values (online supplementary figure 2) and decreased intercept by LD score regression (from 1.086 to 1.04), yet-to-be-determined polygenic effects seem to underlie this disease. We then analysed whether heritability enrichment considering polygenic effects was observed in specific cell groups. As a result, we found that haematopoietic cell groups showed strong enrichment of heritability (p=1.2 × 10⁻⁸; online supplementary figure 3). We further took advantage of LD scores of specific cell types and found strong enrichment of histone marks, especially H3K4me1, of immune-relevant cells including CD56⁺, CD3⁺, CD4⁺ memory and CD8⁺ memory cells (online supplementary table 5 and online supplementary figure 4).

In order to assess important molecular pathways supported by the genetic findings, we conducted pathway analysis using PASCAL software which takes non-significant signals into account. As a result, in addition to interferon (IFN) signalling pathway, other immune-related pathways showed significant associations (online supplementary table 6). It is interesting that some immune-related molecules, including IL-12 and IL-10, were pinpointed.

We further dig into the AHNAK2/PLD4 region. Previous Japanese cell-specific expression quantitative trait loci (eQTL) study did not pinpoint one of the two genes. We decided to focus on PLD4 gene for functional analysis rather than AHNAK2 because of the following reasons. First, PLD2, a family member gene of PLD4, is a susceptibility gene to SLE in the European population. Second, PLD4 regulates kidney fibrosis in human and mice, which is a severe complication of SLE. Third, PLD4 is dominantly expressed in immune-related organs and cells including B cells, monocytes and dendritic cells. Fourth, the previous GWAS meta-analysis of RA in the Japanese population showed a SNP in PLD4 as a top signal in this locus.

Therefore, we have analysed the function of PLD4 using Pld4 mutant mice which was first identified and maintained in Jackson Laboratory. The mutant mice carry a single-nucleotide transition from G to T at position 114 001 632 (NCBI build 37) which is a non-sense mutation, resulting in the introduction of a premature stop codon at residue 46 of the 503 amino acid protein (figure 3A).

We compared mutant homozygous mice (mutant mice, m/m) with mutant heterozygous mice (w/m) in the characterisation analysis. As Jackson Laboratory reported (BH, Submission) mutant mice have revealed decreased weight in comparison with heterozygous mice which became evident soon after birth (figure 3). Adult mutant mice showed marked splenomegaly and lymphadenopathy (figure 3C), inversely proportional to their body size, while the other major non-lymphoid organs were in proportion (online supplementary figure 5). Regarding the number of splenocytes, there was approximately 1.5-fold to 2-fold more in mutant mice compared with heterozygotes (figure 3D).

Microscopic histological analyses revealed remarkable spontaneous formation of germinal centre in spleen and lymphoid organs in mutant mice (figure 3E, arrows; online supplementary figure 6), suggesting the involvement of Pld4 in immunological functions especially in the context of B-cell activities. All experiments were performed using female mice on the assumption that female was more prone to develop autoimmune. However, small body and splenomegaly were observed in male homozygous mutant mice as well.

In order to characterise the lymphoid enlargement, we analysed the immune cell profile within lymphoid organs. Mutant mice bear more T and B cells in the spleen, but cell fraction was comparable (figure 4A). The T cells of mutant mice displayed an activated phenotype, shown as elevated percentage of CD69⁺ cells (figure 4A, right panel). Furthermore, the cell fractions of marginal zone B cells, macrophages and plasmacytoid dendritic cells, which is important for type I IFN secretion, were increased in mutant mice (figure 4B). These results collectively suggest that Pld4 mutant mice has altered the homeostasis of lymphoid organs. Histologically, although most organs of mice 12–15 weeks old showed normal appearance in H&E staining (online supplementary figure 7), some mutant homozygotes showed scattered infiltration of inflammatory cells within the liver (online supplementary figure 7).

Since expansion and activation of B cells were suggested in mutant mice, we analysed B cells in detail. The peripheral B-cell counts in the mutants were proportional to their small body at neonatal period (1 week of age). After 3 weeks of age, the cell numbers rapidly increased, exceeding the number observed in control mouse (online supplementary figure 8A). However, we did not find much difference in the percentage of proliferation of B cells in vitro between the two genotypic groups on anti-IgM, LPS, CpG ODN stimuli (online supplementary figure 8B). We found escalated percentage of germinatal centre B cells and plasma cells (CD138⁺) in the mutant mice (figure 4C). We quantified serum levels of B cell–related molecules and identified hypergammaglobulinemia and marked elevation of BAFF in mutant mice (figure 4C). We did not find class-specific increase in immunoglobulin (figure 4C), suggesting an expansion of plasma cells irrespective of class switch, and possibly, somatic hypermutation. We also found an increase of ANA and anti-DNA antibody in mutant mice (figure 4D).

Since splenomegaly, lymphadenopathy, hypergammaglobulinemia and the increase of ANA and anti-dsDNA antibody suggest B-cell autoimmunity, we assessed SLE-related phenotypes in the mutant mice. We surveyed immune deposition in kidneys in the two genotypic groups. We found PAS-positive deposition in the glomeruli of the mutant mice, but not in the heterozygous mice (figure 4E). Immunohistochemical staining revealed deposition of IgG and C3 in the glomeruli of the mutant mice, but not in the heterozygous mice (figure 4E). In addition, we found upregulation in the gene expression of proinflammatory cytokines (Il6, Il12b) in the spleen and interferon signature in the kidney of mutant mice (lsg15, Oas2, Ifnr4 and Ifna) (online supplementary figure 9) (figure 4E, lower panel). On the contrary, we did not find a remarkable difference of urine protein adjusted for creatinine between the mutant mice and heterozygous mice (figure 4E), and no significant differences were observed regarding lifespan of mutant and heterozygous mice (data not shown).

Collectively, these findings in mice indicate Pld4 involvement in contributing to autoimmunity phenotypes, especially in the production of antinuclear antibody, and immune complex–mediated tissue injury.
**DISCUSSION**

In the current study, we conducted a meta-analysis of GWAS for SLE in the Japanese population and identified a novel susceptibility gene and another susceptibility gene which was very recently reported by a Chinese group. Involvement of **PLD4** in B-cell autoimmunity was supported by analysis using Pld4 mutant mice.

To the best of our knowledge, the association of **PLD4** and **MAMLD1** with SLE in the current study had not been reported in Europeans or in Asian meta-analysis before late 2017. This suggests that heterogeneity exists in the same population group and that there are potential undetermined signals which are specific to a population. Apparently, trans-ethnic meta-analysis is a promising approach to find novel signals by leveraging increased sample sizes, but our results suggest the usefulness of GWAS in a single and relative uniform population. Taking advantage of the difference in LD structure and allele frequencies between populations would be a promising approach to find novel susceptibility loci.

Our analysis also suggests many unknown variants with small effect sizes associated with SLE. Increase of sample sizes in a single population, Asian populations and trans-ethnic populations would lead to identification of novel signals. Our results also identified two suggestive loci as candidates of future replication studies.

The current analyses demonstrated promising pathways and immune cells which would serve as therapeutic targets. Designing clinical and basic experiments based on the list would be an efficient approach for the development of novel treatment for SLE.

While we prefer **PLD4** as a better candidate, **AHNAK2**, the adjacent gene in the same LD block, showed upregulated expression in murine lupus nephritis. There is also a possibility of a trans-eQTL effect of a variant beyond this LD block. Thus, further investigations are needed to pinpoint a mechanism of the association in this locus.

Mammalian PLD is known to be a major player in the regulation of actin cytoskeleton, vesicle trafficking for secretion and endocytosis, and receptor signalling. Proteomics data suggest that human PLD4 is expressed in plasma, monocytes, B lymphocytes and natural killer cells. Within the cells, PLD4 seems to localise mostly to the ER and Golgi, as well as the nucleus. We previously reported that PLD4 is also associated with systemic sclerosis (SSc). Since previous studies have shown overlapping susceptibility genes across multiple autoimmune disorders, PLD4 may have associations with a wide range of autoimmune diseases. rs2841280, a missense variant in **PLD4**, was suggested...
Figure 4  Pld4 mutant mice demonstrate autoimmune phenotypes including production of anti-DNA antibody. (A) Pld4 mutant mice reveal increased T cells with expression of cell surface molecule of activated phenotype. (B) Pld4 mutant mice reveal increased follicular T cell (Fol), marginal zone T cell (MZ), macrophage and plasmacytoid dendritic cell (pDC). cDC, conventional dendritic cell. Cell numbers of Fol, MZ (left panel), macrophages (middle panel) and pDC and cDC are compared between mutant mice and heterozygote mice. (C) Increased B cells and hypergammaglobulinemia and serum BAFF levels in Pld4 mutant mice. B cells in the spleen were analysed by flow cytometry to compare the numbers of germinal centre B cells (CD19+CD95+GL-7+) and plasma cells (B220dim CD138+). Serum concentration of various subclasses of immunoglobulin (age 14–15 weeks) was measured (N=5–9). Circulating BAFF levels were compared between m/w and m/m mice. (D) Autoantibody production in Pld4 mutant mice. Higher levels of anti-dsDNA antibody (left panel) and anti-nuclear antibody (ANA) (right panel) are observed in Pld4 mutant mice. Mef cells were used for ANA quantification. (E) PAS-positive deposition and accumulated IgG and C3 were found in glomeluri in Pld4 mutant mice (upper panel). Interferon-signature genes were highly expressed in the kidney of Pld4 mutant mice (left lower panel). No proteinuria was observed in Pld4 mutant mice (right lower panel). Mutant mice are indicated in dark closed circles or bars. Heterozygous mice are indicated in closed circles or bars. *Indicates p values less than 0.05. †Indicates p values less than 0.01.

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as a candidate causative variant of SSc. This SNP is in LD with rs2582511 (top marker in this study) and showed a significant association in the current study ($r^2=0.71$, $p=3.3 \times 10^{-9}$). These findings suggest that functional alteration of PLD4 due to conformational change with or without change of PLD4 expression would lead to immunological abnormalities. A previous study using Pld4KO mice showed that Pld4 was expressed mainly in the marginal zone of spleen. The same group also showed that Pld4 is implicated in the proliferation and phagocytosis function of microglia in CNS. Collectively, these findings strongly indicate functional involvement of PLD4 in the development of autoimmunity through controlling functions of immune cells.

Based on the significant intracase association between PLD4 risk allele and the production of anti-dsDNA antibody, the risk allele seems to contribute to SLE pathophysiology through activating B cells. Despite the lack of significant associations, the trend of the positive congruence between other antibodies and the PLD4 risk allele may support the hypothesis.

The Pld4 mutant mice revealed remarkably low birth weight and autoimmune phenotypes compatible with SLE, including splenomegaly, lymphadenopathy, expansion of B cells, hypersecretion of BAFF and production of autoantibodies especially ANA and anti-dsDNA antibody. Despite abnormal findings of renal tissues in the mutant mice, they did not show proteinuria and short lives, suggesting that another genetic hit might be necessary to lead to lupus nephritis and renal phenotypes compatible with SLE.

Premature stop codon of Pld4 in the mutant would lead to truncated PLD4 protein. Since all the cells in the mutant mice harbour this gene mutation, our study could not clarify whether
the gene mutation has a direct effect on specific immune cell types or has resulted in indirect phenotypes due to mutations of non-haematopoietic cells. Despite the above limitation, taking into consideration that peripheral B-cell subpopulations but not B-cell precursors within the bone marrow (data not shown) were altered in mutant mice, Plld4 seems to affect peripheral maturation or survival of B cells. Lineage-specific ablation of Plld4 or induction of the mutation would clarify cellular designation to phenotypes in mutant mice.

We have not elucidated the detailed mechanism into how the truncated Plld4 protein or lack of normal Plld4 itself could lead to low body weight and immune dysfunction especially for acquired immunity. The latest report revealed Plld4-deficient mice demonstrated exaggerated TLR9 response and spontaneous inflammation.\(^\text{30}\) This dysfunction of immune system may at least partly explain phenotypes found in the current study.

Mammalian MAMLD1 encodes a mastermind-like domain containing protein. Human X-linked hypsolesplasia type 2 is known to be caused by a loss-of-function mutation in this gene.\(^\text{23}\) Due to lack of evidence of eQTL association and amino acid alteration of rs143181706 on MAMLD1, we could not infer how the variant and/or gene would contribute to SLE susceptibility. Since MAMLD1 is associated with sex development, MAMLD1 association might be related to high frequency of women in patients with lupus. The positive associations between MAMLD1 risk allele and autoantibody production may suggest MAMLD1 function on B cells. Further in vivo analysis would clarify these points.

Taken together, we identified a novel susceptibility locus to SLE and conducted in vivo functional study of PLD4. These genes and encoding proteins may serve as candidates of cellular and molecular targets in SLE. Further increase of sample sizes in the study subjects for GWAS would uncover genetic susceptibility loci to SLE. It would be interesting to develop mutant mice carrying multiple mutations of SLE-susceptibility genes.

Acknowledgements  We would like to thank all the medical and technical staff for helping us to collect DNA samples.

Contributors  Wrote the paper: SA, S-ML, CT. Data analysis: SA, KJ, CT. Performed the mouse experimental work: SA, YuK, S-ML, AS, MN, YI, HK, OJ, JH. CT. Conceived and designed the study: CT. Substantial contribution to acquired samples and creation of data in 1st GWAS: KM, KO, FM, YO, TM, CT. Substantial contribution to acquired samples and creation of data in 2nd GWAS: KJ, YuK, YOK, TS, KY, YO. Authors revised and approved the manuscript to be published.

Funding  This study was supported by JSPS KAKENHI (grant nos. JP16H06251 and 16K08981), Nagao Memorial Fund and The Kato Memorial Trust for Nanmyo Research.

Competing interests  None declared.

Patient consent for publication  Obtained.

Ethics approval  This study was approved by the ethical committee in each institution.

Provenance and peer review  Not commissioned; externally peer reviewed.

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