Title:

Autotaxin may play a Critical Role between the Genetic Risk Factors and Pathogenesis of SLE in Plasmacytoid Dendritic Cells

Authors:

Yumi Tsuchida[1], Hirofumi Shoda[1], Masahiro Nakano[1,2], Mineto Ota[1,3], Tomohisa Okamura[1,3], Kazuhiko Yamamoto[1,2], Makoto Kurano[4], Yutaka Yatomi[4], Keishi Fujio[1*], Tetsuji Sawada[5]

Affiliations:

[1]Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

[2] Laboratory for Autoimmune Diseases, RIKEN Center for Integrative Medical Sciences, Yokohama, Japan.

[3] Department of Functional Genomics and Immunological Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

[4] Department of Clinical Laboratory Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan.

[5] Department of Rheumatology, Tokyo Medical University Hospital, Tokyo, Japan.

Corresponding Author:

Keishi Fujio M.D., Ph.D.

Department of Allergy and Rheumatology, Graduate School of Medicine, The University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

Phone number: +81-3-3815-5411

Fax number: +81-3-3815-5954

E-mail address: FUJIOK-INT@h.u-tokyo.ac.jp

Abstract

The importance of autotaxin, which catalyzes the production of lysophospholipids, has recently been recognized in various diseases including cancer and autoimmune diseases. We herein report our analysis of autotaxin in systemic lupus erythematosus (SLE), utilizing data from ImmuNexUT, a comprehensive database consisting of transcriptome data and expression quantitative trait locus (eQTL) data of immune cells from patients with immune-mediated disorders. Autotaxin was elevated in the serum of SLE patients, and the expression of *ENPP2*, which encodes autotaxin, is elevated in plasmacytoid dendritic cells (pDCs) of SLE patients compared to healthy controls. In weighted correlation network analysis, *ENPP2* belonged to a module that correlated with disease activity. This module was enriched in interferon-associated genes and included genes whose expression is influenced by SNPs associated with SLE, suggesting that it is a key module connecting genetic risk factors of SLE with disease pathogenesis. The increased expression of *ENPP2* in pDCs from SLE patients may be due to increased expression of interferon-associated genes and increased binding of STAT3 complexes to the regulatory region of *ENPP2*. Thus, autotaxin may play a critical role in connecting genetic risk factors of SLE to disease pathogenesis in pDCs.

Introduction

Lysophospholipids (LPLs) are phospholipids with only one fatty acid chain, including Sphingosine-1-Phosphate (S1P), lysophosphatidic acids (LPA), and lysophosphatidylserine. By signaling through their respective G protein-coupled receptors, they play important roles in various biological processes[1]. Recently, enzymes that catalyze the formation of those LPLs, such as autotaxin and phosphatidylserine-specific phospholipase A1 (PS-PLA1), have been reported as potential biomarkers of various diseases. For example, serum autotaxin levels and PS-PLA1 levels have been associated with melanoma[2]. Autotaxin and PS-PLA1 have also been associated with immune mediated diseases. For example, the level of PS-PLA1 and autotaxin are elevated in the serum of patients with lupus nephritis compared patients with other glomerular diseases[3], and the level of PS-PLA1 in the serum correlates with disease activity in systemic lupus erythematosus (SLE) patients[4]. Also, it has been reported that autotaxin is increased in the urine of patients with lupus nephritis[5]. Therefore, autotoxin could be important in the pathogenesis of SLE; however, the exact role of autotaxin in the pathogenesis of SLE remains unclear.

Both genetic and environmental factors contribute to the development of SLE. Various genome wide association studies have been performed to identify genetic risk

factors for SLE, and more than 100 loci have been reported to be associated with SLE[6]. However, the exact mechanism by which those GWAS SNPs contribute to the development of SLE in often not clear, and further studies are necessary to elucidate the link between genetic risk factors of SLE and disease pathogenesis and to develop new treatment strategies.

Recently, our group has created a transcriptome and eQTL database of immune cells from various immune-mediated diseases, "ImmuNexUT" [7]. This database consists of the genome and transcriptome of 28 immune cells from patients with various immune-mediated diseases, as well as healthy controls (HC). Here, to further elucidate the pathogenic role of autotaxin in SLE, we utilized the data from ImmuNexUT[7] to examine the expression pattern of autotaxin in immune cells, its relationship to clinical parameters, and genetic risk factors of SLE.

Results

Autotaxin is elevated in the serum of SLE patients

First, we examined the concentration of autotaxin in the serum of untreated SLE patients and HC. As the serum concentration of autotaxin is reported to be different between females and males[8], females and males were analyzed separately. The serum

concentration of autotaxin was higher in SLE patients compared to HC in both females (Fgure 1a) and males (Figure 1b).

The expression of ENPP2 is elevated in plasmacytoid dendritic cells of SLE patients

Next, we utilized the transcriptome data of SLE patients and age and sex matched HC from ImmuNexUT to examine the mRNA expression level of *ENPP2*, which encodes autotaxin. The expression of *ENPP2* was high in plasmacytoid dendritic cells (pDCs) compared to other immune cell subsets, as reported previously[7,9] (Figure 2a). Therefore, we focused on pDCs, and the expression of *ENPP2* was elevated in pDCs of SLE patients compared to HC, as noted by others[9] (Figure 2b). When the transcriptome of pDCs from females and males were analyzed separately, *ENPP2* was also identified as differentially expressed gene (DEG) in both females (Figure 2c) and males (Figure 2d).

ENPP2 is co-expressed with interferon-associated genes

To further examine the role of *ENPP2* in the pathogenesis of SLE, weighted correlation network analysis (WGCNA) was performed to group genes into modules of genes with similar expression patterns, using RNA-seq data of pDCs from SLE patients.

(Figure 3a). *ENPP2* belonged to the magenta module (Supplementary Table S2), and the eigengene score of this module showed correlation with SLEDAI-2K, as well as anti-RNP antibody positivity (Figure 3a). The eigengene score of this module was high in patients with moderate or high disease activity (SLEDAI-2K > 6) compared to patients with mild or no disease activity (SLEDAI-2K ≤ 6) (Figure 3b). The magenta module included many interferon-associated genes, such as *IFIH1* and *ISG15*, and pathway analysis of the genes in this module showed enrichment of interferon-associated pathways (Figure 3c).

The magenta module links genetic risk factors of SLE with the pathogenesis of SLE

Further examination of the members of the magenta module using the eQTL database from ImmuNexUT[7] indicated that this module contains genes whose expression is influenced by SLE GWAS SNPs in pDCs. For example, rs13385731, in LD with SLE GWAS SNP rs13425999[10], is an expression quantitative trait locus (eQTL) of *RASGRP3* in pDCs (Figure 4a). That is, in patients with the SLE risk allele, the expression of *RASGRP3* is increased in pDCs. In addition, rs11059921, in LD with SLE GWA SNP rs11059919[11], influences the expression of *SLC15A4*, suggesting that this module may reflect the link between genetic risk factor of SLE and disease pathogenesis (Figure 4b).

rs11778951 influences the expression of ENPP2 in pDCs

Next, to elucidate the mechanism for the increased expression of *ENPP2* in pDC of SLE patients, we examined the eQTL database in ImmuNexUT. rs11778951, located in the *ENPP2* gene, influenced the expression of *ENPP2* in pDCs with the G allele increasing the expression of *ENPP2* (Figure 5a). ATAC-seq data of immune cells from a public database[12] indicated that rs11778951 coincides with a peak specific in pDCs (Figure 5b). This locus was predicted to be a distal enhancer-like signature in ENCODE[13] and resided near predicted IRF1 and STAT3 binding sites[14]. Furthermore, rs11778951 alters a STAT binding motif at this site[15]. Therefore, the increase in interferon signaling in SLE patients may cause increased binding of STAT3 complexes to this site in pDCs, leading to the increased expression of *ENPP2*.

Discussion

We herein describe our analysis of autotaxin and related pathways using the ImmuNexUT database. Autotaxin is elevated in the serum of SLE patients, and the expression of *ENPP2*, encoding autotaxin, is increased in pDCs of SLE patients. WGCNA analysis indicated that the expression of *ENPP2* correlates with the magenta module,

whose members are enriched in interferon-associated genes and whose expression correlates with disease activity of SLE. The magenta module included genes whose expression are affected by SLE GWAS SNPs, suggesting that *ENPP2* may be one of the key molecules linking genetic risk factors of SLE with disease pathogenesis. Furthermore, rs11778951, which coincides with an ATAC-seq peak specific to pDCs, alters STAT protein binding motifs, has an eQTL effect on *ENPP2*, suggesting that the increased interferon signaling in SLE patients may contribute to the increased expression of *ENPP2* in SLE.

This study has suggested important points regarding the role of autotaxin in SLE. It has been reported that autotaxin is highly expressed in adipose tissue, central nervous system, and reproductive organs[16], and in the immune system, autotaxin is expressed by endothelial cells in high endothelial venules. Dendric cells has not generally gained much attention as an important source of autotaxin; however, a recent report regarding the role of autotaxin in COVID19 has suggested that autotaxin expressed by pDC may affect the development and function of DCs by regulating the local production of LPA[9]. Our study suggests that the increased expression of autotaxin in pDCs of SLE patients may contribute to pathophysiology of SLE through altered DC function, as well.

Our study indicated that the expression of interferon associated genes show

correlation with the expression of *ENPP2* in pDCs. A role for type I interferons in the production of autotaxin has previously reported[17], and our results are consistent with this. Furthermore, by combining WGCNA analysis with eQTL database, a potential mechanism for increased expression of *ENPP2* in pDCS in SLE patients was identified. That is, the increase in interferon-signaling induced by genetic risk factor of SLE may lead to the increased expression of *ENPP2* in pDCs by increasing the binding of STAT proteins to rs11778951, an eQTL locus of *ENPP2* in pDCs.

In conclusion, our transcriptome analysis of pDC identified a gene module, whose expression correlates with disease activity in SLE patients and is affected by SLE GWAS SNPs. By examining genes in this module, autotaxin expressed by pDC was identified as a possible key molecule in the link between genetic risk factors and pathogenesis of SLE.

Methods

Autotaxin ELISA

Serum samples were obtained from 54 patients with active, untreated SLE (42 women, 12 men) who fulfilled the 1997 American College of Rheumatology (ACR) revised classification criteria at the University of Tokyo Hospital and the Tokyo Medical University Hospital. Serum samples were also collected from 237 healthy individuals (69 women and 168 men).

Serum autotaxin was quantified by one-step immunoenzymometric assay, as described elsewhere[8]. In brief, a pair of rat monoclonal antibodies (mAb) specific for human autotaxin was used for the two-site immunoassay. The autotaxin immunoassay reagents containing magnetic polymer beads coated with the primary mAb, and an alkaline phosphatase-labeled secondary mAb were placed in the reaction cup. After addition of diluted serum sample into the reaction cup, the resulting assay reagent was applied to a commercial automated immunoassay analyzer (AIA-2000 system; Tosoh, Tokyo, Japan).

Differential expression analysis

For the differential gene expression analysis, RNA-seq data from the

ImmuNexUT study[7] was utilized. Details of the sample collection, RNA-sequencing, and data processing have been described in detail previously[7]. SLE patients with expression data for pDCs after filtering were included (n=59), and 51 healthy controls, age and gender matched to the SLE patients, were selected as controls. The SLE patients fulfilled the American College of Rheumatology (ACR) 1997 classification criteria[18] and SLICC 2012 criteria[19]. Patients with malignancies, acute infections, or those taking with more than 20 mg prednisolone were excluded. Disease activity was assessed with SLEDAI-2K[20]. Differential expression analysis was performed with edgeR after TMM normalization[21].

WGCNA analysis

For weighted gene co-expression network analysis (WGCNA) analysis, genes with read counts less than 10 in 90% or more of the samples were excluded. Count data was normalized with TMM normalization and converted to log 2 (CPM + 1). WGCNA was performed using WGCNA package version 1.68 with signed network and soft thresholding power set to 8. One sample was excluded as an outlier. The representative expression value of each module, “eigengenes”, were calculated, and the correlation between eigengenes and clinical parameters were assessed. Pathway analysis was

performed by DAVID[22] using REACTOME pathways[23].

eQTL analysis

For the eQTL analysis, the eQTL data from ImmNexUT database were utilized, and the details are described in the initial study[7]. After filtering out genes expressed with low expression in each cell subset, the expression data were normalized with TMM normalization, converted to CPM and normalized across samples using an inverse normal transform. Probabilistic Estimation of Expression Residuals (PEER) method [24] was used to find hidden covariates. An QTLtools permutation pass with 10,000 permutations was used to obtain gene-level nominal P value thresholds corresponding to FDR < 0.05 for each cell subset. Then, a forward-backward stepwise regression eQTL analysis was performed with a QTLtools conditional pass. ATAC-seq data from the report by Calderon et al.[12] was used to assess the relationship of eQTL SNPs with ATAC-seq peaks. Data regarding candidate cis-regulatory elements from the ENCODE study was utilized for the visualization of distal enhancer like signatures[25], and data from JASPAR[14] was used to visualize predicted transcription factor binding sites.

Ethics declarations

The ethics committees at the University of Tokyo Hospital and the Tokyo Medical University Hospital approved this study, and written consent was obtained from all participants (G10095).

Statistics

Categorical data were tested with Fisher's exact test. Differences between two groups were compared with t-tests for normally-distributed continuous data and with Mann-Whitney U tests for non-normally distributed continuous data. Correlations were evaluated with Spearman's rank correlation coefficients. P values less than 0.05 were considered significant. Statistical analyses were performed with R version 3.5.0 (R Foundation for Statistical Computing).

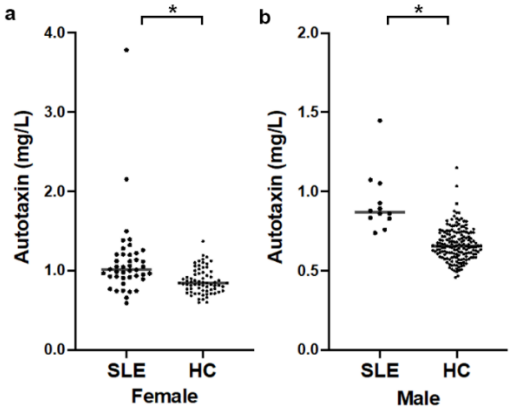

Figure 1. Autotaxin is elevated in the serum of SLE patients.

Serum autotaxin levels in (a) female and (b) male SLE patients and HC.

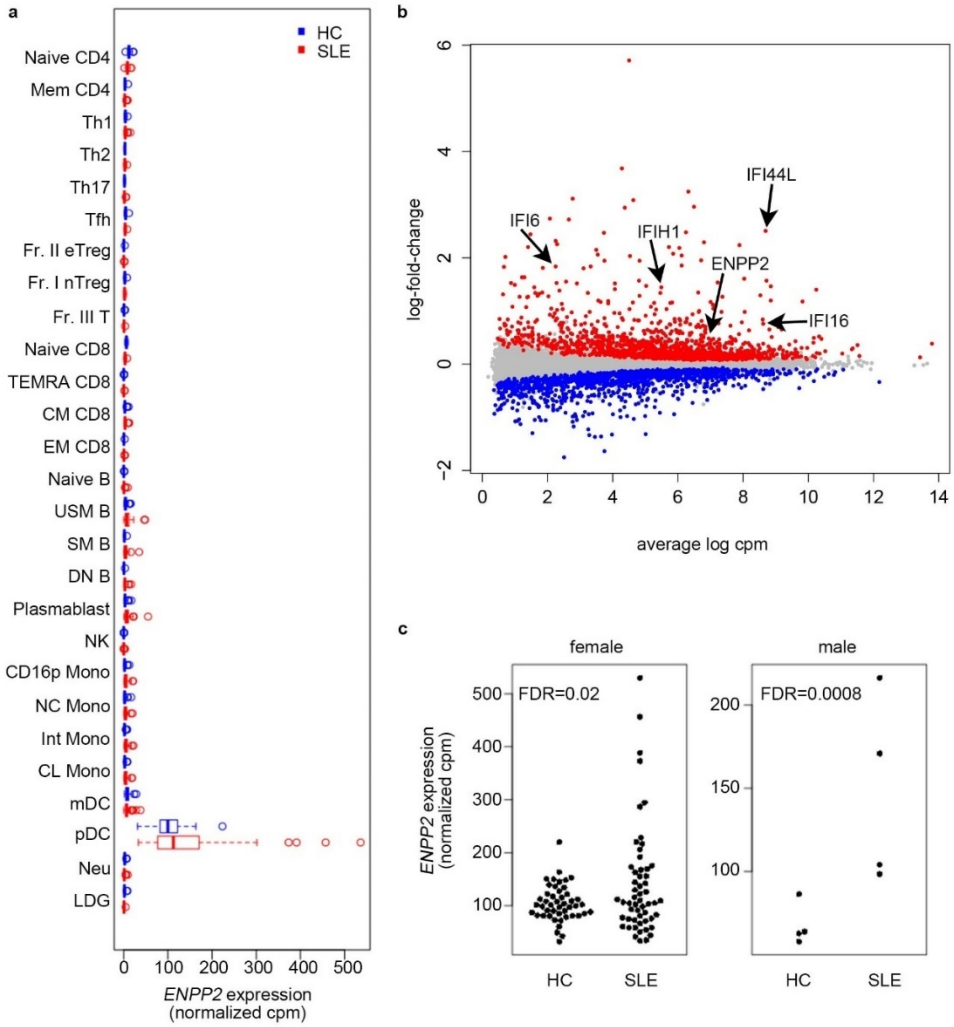

Figure 2. The expression of *ENPP2* is elevated in pDCs of SLE patients.

(a) Expression of *ENPP2* in immune cell subsets of SLE patients and HC. For abbreviations, see supplementary table S1.

(b) MA plot of pDCs from SLE patients and HC with selected genes highlighted.

(c) *ENPP2* expression in pDCs from HC and SLE patients.

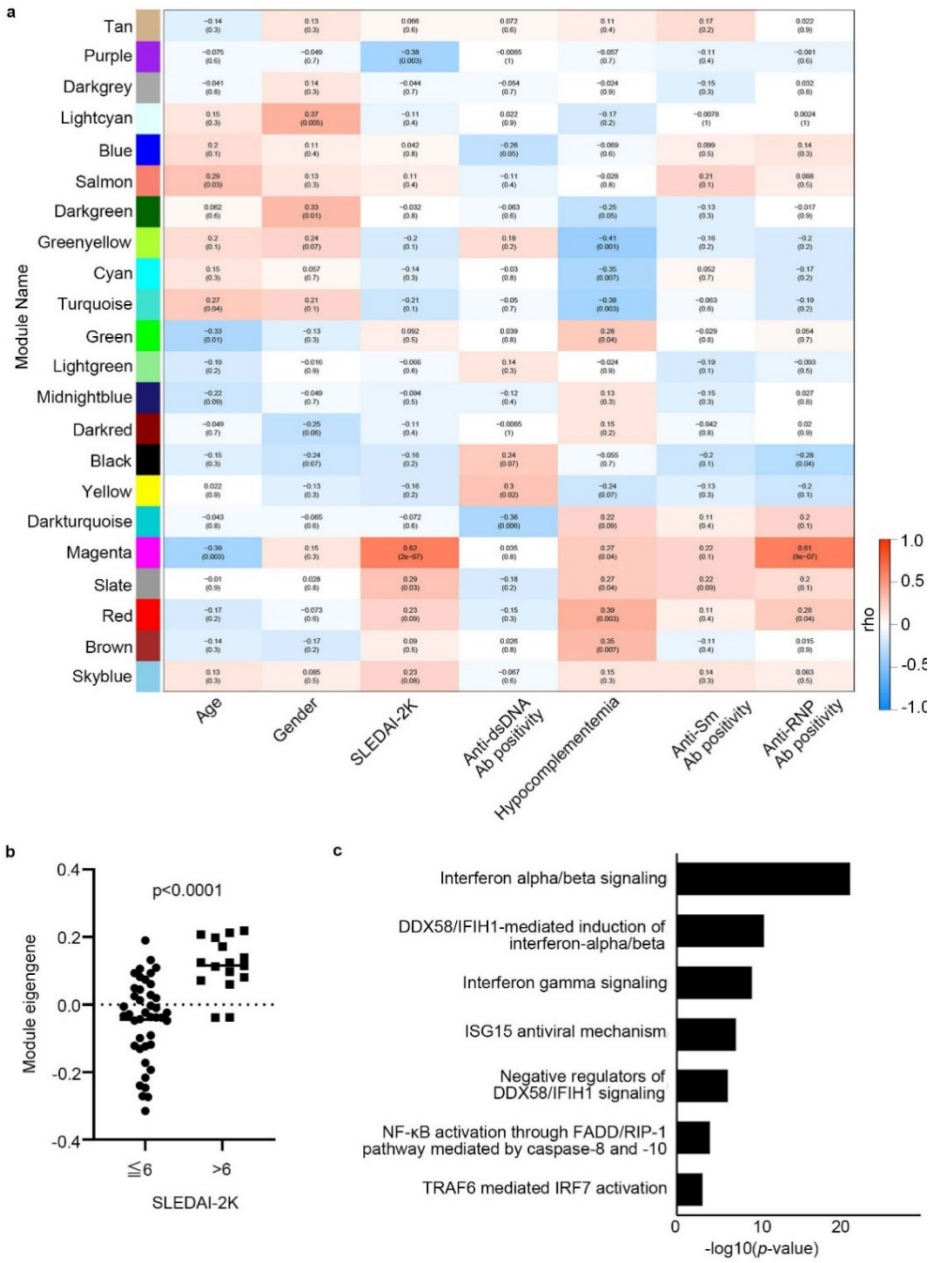

Figure 3. *ENPP2* is co-expressed with interferon-associated genes.

(a) Correlation of WGCNA modules with clinical parameters. In each box, the numbers in the upper row indicate the correlation coefficient, and the numbers in the bottom row indicate the *p*-value.

(b) Module eigengenes of the magenta module in SLE patients with SLEDAI-2K scores ≤ 6 and > 6.

(c) Pathway analysis of the members of the magenta module. The enrich

(d) ment of the genes in the magenta module for REACTOME pathways was tested using Fisher's exact test.

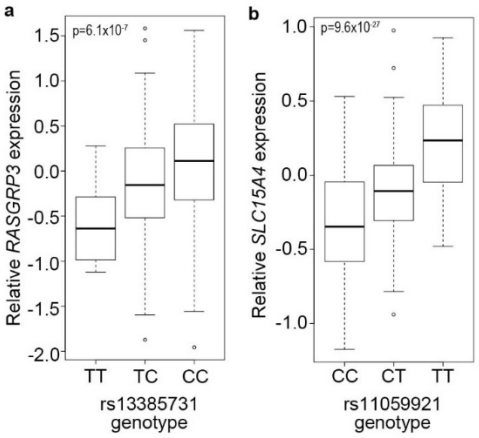

Figure 4. The magenta module links genetic risk factors of SLE with the pathogenesis of SLE.

(a) eQTL effect of rs13385731 on *RASGRP3.*

(b) eQTL effect of rs11059921 on *SLC15A4*.

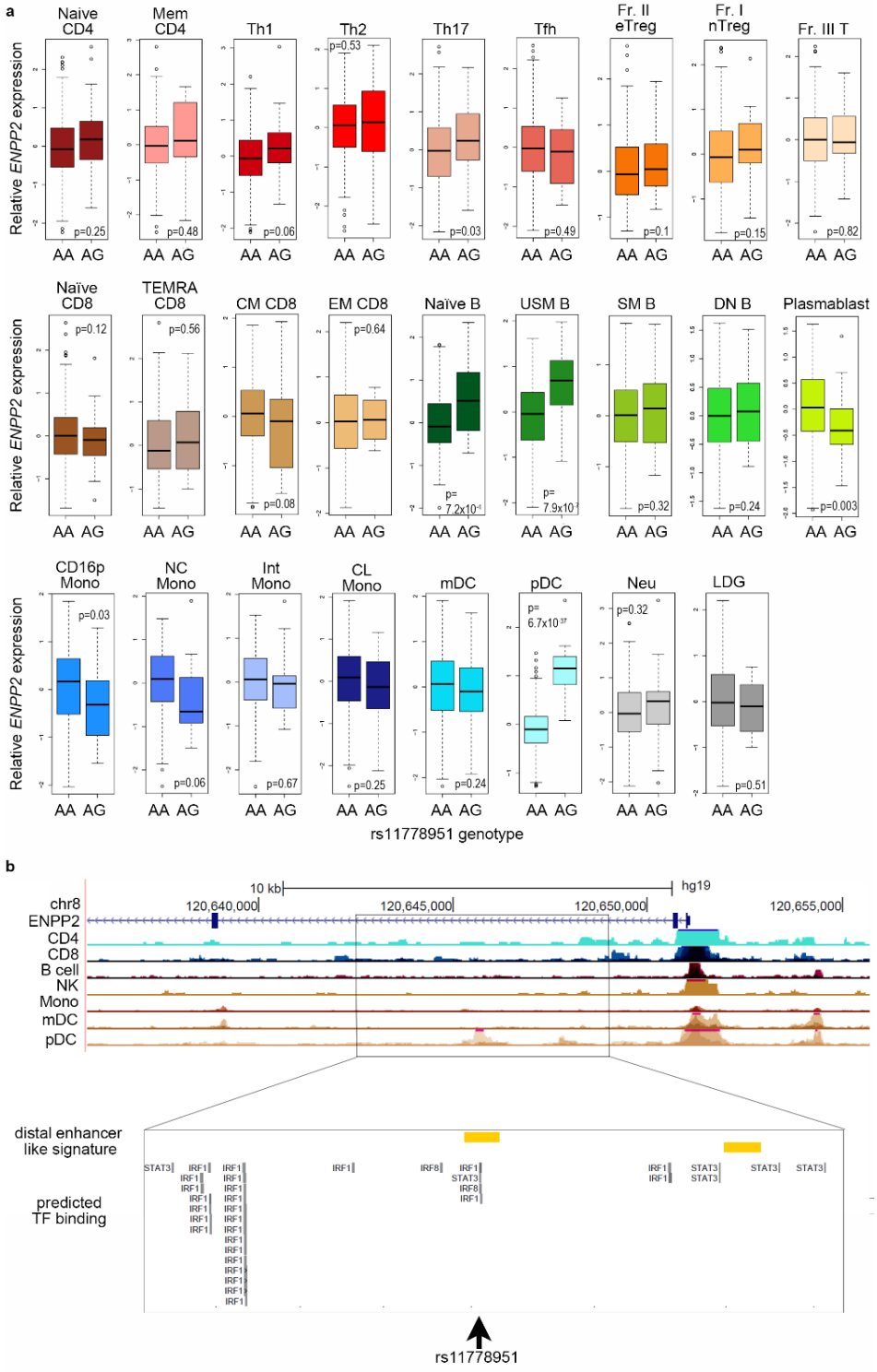

Figure 5. rs11778951 influences the expression of *ENPP2* in pDCs.

(a) The expression of *ENPP2* by rs11778951 genotype.

(b) ATAC-seq peaks near rs11778951 are shown, along with distal enhancer like signatures and predicted transcription factor (TF) binding sites.

Acknowledgements
We would like to express our gratitude to all the study participants for their cooperation for this study. The supercomputing resource, SHIROKANE, was provided by the Human Genome Center, The University of Tokyo. This study was supported by Chugai Pharmaceutical Co., Ltd.

Author contributions
Y.T. performed the DEG analysis and wrote the manuscript with H.S. M.N. performed the WGCNA analysis. M.O. performed the eQTL analysis. T.S. performed the ELISA with M.K. and Y.Y. T.O., K.Y., K.F., and T.S. supervised the study. T.S. and K.F. conceptualized the work.

Competing interests
T.O. and M.O. belong to the Social Cooperation Program, Department of Functional Genomics and Immunological Diseases, supported by Chugai Pharmaceutical. K.F. receives consulting honoraria and research support from Chugai Pharmaceutical.

Data availability
The RNA-seq data is available at the National Bioscience Database Center (NBDC) (E-GEAD-397).

References

1. Gräler, M. H. & Goetzl, E. J. Lysophospholipids and their G protein-coupled receptors in inflammation and immunity. *Biochim Biophys Acta* **1582**, 168-174, doi:10.1016/s1388-1981(02)00152-x (2002).
2. Kurano, M. *et al.* Association between serum autotaxin or phosphatidylserine-specific phospholipase A1 levels and melanoma. *J Dermatol* **45**, 571-579, doi:10.1111/1346-8138.14278 (2018).
3. Iwata, Y. *et al.* Higher serum levels of autotaxin and phosphatidylserine-specific phospholipase A(1) in patients with lupus nephritis. *Int J Rheum Dis* **24**, 231-239, doi:10.1111/1756-185x.14031 (2021).
4. Sawada, T. *et al.* Serum phosphatidylserine-specific phospholipase A(1) as a novel biomarker for monitoring systemic lupus erythematosus disease activity. *Int J Rheum Dis* **22**, 2059-2066, doi:10.1111/1756-185x.13689 (2019).
5. Morita, Y. *et al.* Urinary autotaxin concentrations are associated with kidney injury. *Clin Chim Acta* **509**, 156-165, doi:10.1016/j.cca.2020.06.019 (2020).
6. Fike, A. J., Elcheva, I. & Rahman, Z. S. M. The Post-GWAS Era: How to Validate the Contribution of Gene Variants in Lupus. *Curr Rheumatol Rep* **21**, 3, doi:10.1007/s11926-019-0801-5 (2019).
7. Ota, M. *et al.* Dynamic landscape of immune cell-specific gene regulation in immune-mediated diseases. *Cell* **184**, 3006-3021.e3017, doi:10.1016/j.cell.2021.03.056 (2021).
8. Nakamura, K. *et al.* Validation of an autotaxin enzyme immunoassay in human serum samples and its application to hypoalbuminemia differentiation. *Clin Chim Acta* **388**, 51-58, doi:10.1016/j.cca.2007.10.005 (2008).
9. Nikitopoulou, I. *et al.* Increased Autotaxin Levels in Severe COVID-19, Correlating with IL-6 Levels, Endothelial Dysfunction Biomarkers, and Impaired Functions of Dendritic Cells. *Int J Mol Sci* **22**, doi:10.3390/ijms221810006 (2021).
10. Molineros, J. E. *et al.* Evaluation of SLE Susceptibility Genes in Malaysians. *Autoimmune Dis* **2014**, 305436, doi:10.1155/2014/305436 (2014).
11. Bentham, J. *et al.* Genetic association analyses implicate aberrant regulation of innate and adaptive immunity genes in the pathogenesis of systemic lupus erythematosus. *Nat Genet* **47**, 1457-1464, doi:10.1038/ng.3434 (2015).
12. Calderon, D. *et al.* Landscape of stimulation-responsive chromatin across diverse human immune cells. *Nat Genet* **51**, 1494-1505, doi:10.1038/s41588-019-0505-9 (2019).
13. Moore, J. E. *et al.* Expanded encyclopaedias of DNA elements in the human and mouse genomes. *Nature* **583**, 699-710, doi:10.1038/s41586-020-2493-4 (2020).
14. Fornes, O. *et al.* JASPAR 2020: update of the open-access database of transcription factor

binding profiles. *Nucleic Acids Res* **48**, D87-d92, doi:10.1093/nar/gkz1001 (2020).

15. Ward, L. D. & Kellis, M. HaploReg: a resource for exploring chromatin states, conservation, and regulatory motif alterations within sets of genetically linked variants. *Nucleic Acids Res* **40**, D930-934, doi:10.1093/nar/gkr917 (2012).
16. Magkrioti, C. *et al.* Autotaxin and chronic inflammatory diseases. *J Autoimmun* **104**, 102327, doi:10.1016/j.jaut.2019.102327 (2019).
17. Song, J., Guan, M., Zhao, Z. & Zhang, J. Type I Interferons Function as Autocrine and Paracrine Factors to Induce Autotaxin in Response to TLR Activation. *PLoS One* **10**, e0136629, doi:10.1371/journal.pone.0136629 (2015).
18. Hochberg, M. C. Updating the American College of Rheumatology revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* **40**, 1725, doi:10.1002/art.1780400928 (1997).
19. Bortoluzzi, A., Furini, F., Campanaro, F. & Govoni, M. Application of SLICC classification criteria in undifferentiated connective tissue disease and evolution in systemic lupus erythematosus: analysis of a large monocentric cohort with a long-term follow-up. *Lupus* **26**, 616-622, doi:10.1177/0961203316671814 (2017).
20. Gladman, D. D., Ibañez, D. & Urowitz, M. B. Systemic lupus erythematosus disease activity index 2000. *J Rheumatol* **29**, 288-291 (2002).
21. Robinson, M., McCarthy, D. & Smyth, G. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics (Oxford, England)* **26**, 139-140, doi:10.1093/bioinformatics/btp616 (2010).
22. Jiao, X. *et al.* DAVID-WS: a stateful web service to facilitate gene/protein list analysis. *Bioinformatics (Oxford, England)* **28**, 1805-1806, doi:10.1093/bioinformatics/bts251 (2012).
23. Jassal, B. *et al.* The reactome pathway knowledgebase. *Nucleic Acids Res* **48**, D498-d503, doi:10.1093/nar/gkz1031 (2020).
24. Stegle, O., Parts, L., Piipari, M., Winn, J. & Durbin, R. Using probabilistic estimation of expression residuals (PEER) to obtain increased power and interpretability of gene expression analyses. *Nat. Protoc.* **7**, 500-507, doi:10.1038/nprot.2011.457 (2012).
25. Kheradpour, P. & Kellis, M. Systematic discovery and characterization of regulatory motifs in ENCODE TF binding experiments. *Nucleic Acids Res* **42**, 2976-2987, doi:10.1093/nar/gkt1249 (2014).