

ORIGINAL ARTICLE

Activation of RHOA–VAV1 signaling in angioimmunoblastic T-cell lymphoma

M Fujisawa^{1,17}, M Sakata-Yanagimoto^{1,2,3,17}, S Nishizawa¹, D Komori¹, P Gershon⁴, M Kiryu¹, S Tanzima¹, K Fukumoto¹, T Enami¹, M Muratani⁵, K Yoshida⁶, S Ogawa⁶, K Matsue⁷, N Nakamura⁸, K Takeuchi^{9,10}, K Izutsu^{11,12}, K Fujimoto¹³, T Teshima¹³, H Miyoshi¹⁴, P Gaulard¹⁵, K Ohshima¹⁴ and S Chiba^{1,2,3,16}

Somatic G17V *RHOA* mutations were found in 50–70% of angioimmunoblastic T-cell lymphoma (AITL). The mutant *RHOA* lacks GTP binding capacity, suggesting defects in the classical *RHOA* signaling. Here, we discovered the novel function of the G17V *RHOA*: VAV1 was identified as a G17V *RHOA*-specific binding partner via high-throughput screening. We found that binding of G17V *RHOA* to VAV1 augmented its adaptor function through phosphorylation of 174Tyr, resulting in acceleration of T-cell receptor (TCR) signaling. Enrichment of cytokine and chemokine-related pathways was also evident by the expression of G17V *RHOA*. We further identified VAV1 mutations and a new translocation, *VAV1*–*STAP2*, in seven of the 85 *RHOA* mutation-negative samples (8.2%), whereas none of the 41 *RHOA* mutation-positive samples exhibited VAV1 mutations. Augmentation of 174Tyr phosphorylation was also demonstrated in VAV1–*STAP2*. Dasatinib, a multikinase inhibitor, efficiently blocked the accelerated VAV1 phosphorylation and the associating TCR signaling by both G17V *RHOA* and VAV1–*STAP2* expression. Phospho-VAV1 staining was demonstrated in the clinical specimens harboring G17V *RHOA* and VAV1 mutations at a higher frequency than those without. Our findings indicate that the G17V *RHOA*–VAV1 axis may provide a new therapeutic target in AITL.

Leukemia (2018) 32, 694–702; doi:10.1038/leu.2017.273

INTRODUCTION

Angioimmunoblastic T-cell lymphoma (AITL) is a subtype of peripheral T-cell lymphoma.¹ AITL patients display generalized lymphadenopathy and immune system-mediated manifestations including high fever, skin rash, polyarthritis, hemolytic anemia and hypergammaglobulinemia.² We and others previously conducted gene mutational profiling of AITL samples and observed *RHOA* mutations converting glycine to valine at amino acid 17 (the G17V *RHOA* mutation) in up to 70% of AITL.^{3–5} Genes encoding the epigenetic regulators *TET2*, *DNMT3A* and *IDH2* are also frequently mutated in AITL.^{3,4,6–10}

RHOA is a small GTPase that cycles between guanosine diphosphate (GDP)-bound inactive and guanosine-triphosphate (GTP)-bound active forms. Guanine nucleotide exchange factors (GEFs) activate *RHOA* by replacing GDP with GTP. Physiologically, *RHOA* mediates migration and polarity of T cells.^{11,12} *RHOA* also functions in thymocyte development^{13,14} and activation of pre-T-cell receptor (pre-TCR) signaling in thymocytes.^{13,15} Glycine at *RHOA* residue 17 is located at a critical position for GTP binding. G17V *RHOA* protein is considered to be a loss-of-function mutant, as G17V *RHOA* does not bind Rhotekin, a molecule with high

affinity for the GTP-bound form.^{3–5} Nonetheless, the impact of G17V *RHOA* expression on AITL remains unclear.

The VAV1 protein mediates a signaling cascade triggered by the TCR engagement partly through GEF activity,¹⁶ whereas GEF-independent VAV1 functions are also reported.^{17,18} In the latter, VAV1 functions as an adaptor in a protein complex that promotes phosphorylation of phospholipase C- γ 1 (PLC γ 1).^{18,19}

PLC γ 1 phosphorylation induces its own enzymatic activity to upregulate the second messengers diacylglycerol and inositol 1,4,5-triphosphate, in turn promoting calcium–calmodulin signaling and enhancing nuclear factor of activated T cells (NFAT) transcription.²⁰ VAV1 also functions in extracellular signal-regulated kinase, c-Jun N-terminal kinase and nuclear factor- κ B pathways,²¹ and its activation is tightly regulated by multilayered autoinhibition by interaction of its Dbl-homology (DH) domain with both acidic (167–178)²² and C-terminal Src homology 2 (SH2)/SH3 domains. TCR engagement initially triggers the phosphorylation of Tyr142 and Tyr160 of VAV1 protein, destabilizing modulatory contacts and facilitating recruitment of the Src kinases LCK and FYN by providing a docking site for their SH2 domains. Thereafter, VAV1 Tyr174 is phosphorylated,²³ relieving core inhibitory interactions with the acidic and DH domains,

¹Department of Hematology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, Tsukuba, Japan; ²Department of Hematology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan; ³Department of Hematology, University of Tsukuba Hospital, Tsukuba, Japan; ⁴Department of Molecular Biology and Biochemistry, UC-Irvine, Irvine, CA, USA; ⁵Department of Genome Biology, Faculty of Medicine, University of Tsukuba, Tsukuba, Japan; ⁶Department of Pathology and Tumor Biology, Graduate School of Medicine, Kyoto University, Kyoto, Japan; ⁷Division of Hematology/Oncology, Department of Medicine, Kameda Medical Center, Kamogawa, Japan; ⁸Department of Pathology, Tokai University School of Medicine, Isehara, Japan; ⁹Division of Pathology, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; ¹⁰Pathology Project for Molecular Targets, Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; ¹¹Department of Hematology, Toranomon Hospital, Tokyo, Japan; ¹²Okinaka Memorial Institute for Medical Research, Tokyo, Japan; ¹³Department of Hematology, Hokkaido University Graduate School of Medicine, Sapporo, Japan; ¹⁴Department of Pathology, Kurume University School of Medicine, Kurume, Japan; ¹⁵InsERM U955, Université Paris Est and Département de Pathologie, Hôpital Henri Mondor, Assistance Publique-Hôpitaux de Paris, Créteil, France and ¹⁶Life Science Center of Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba, Japan. Correspondence: Dr M Sakata-Yanagimoto or Professor S Chiba, Department of Hematology, Faculty of Medicine, University of Tsukuba Hospital, 1-1-1, Tsukuba, Ibaraki 305-8575, Japan.

E-mail: sakatama-tyk@umin.net or schiba-tyk@umin.net

¹⁷These two authors contributed equally to this work.

Received 20 February 2017; revised 20 June 2017; accepted 17 July 2017; accepted article preview online 23 August 2017; advance online publication, 22 September 2017

resulting in the activation of downstream effectors. Conversion of Tyr174 to either Phe¹⁷ or Asp²⁴ or physiologic phosphorylation of the wild-type Tyr174 residue reportedly activates VAV1 signaling. In addition, deletion of the VAV1 C terminus enhances its signaling.²⁵

Here we used mass spectrometry and immunoprecipitation to show that the G17V RHOA protein specifically binds to VAV1 protein. Upon TCR stimulation, VAV1 binding to G17V RHOA accelerated VAV1 phosphorylation and the eventual downstream signaling cascade.

MATERIALS AND METHODS

Patients and samples

Samples were obtained from patients with approval of local ethics committees in all participating institutes. Informed consent was obtained from all living subjects.

Cells

Jurkat cells inducibly expressing the wild-type (WT) and G17V RHOA mutant complementary DNA (cDNA) and mock-transduced cells have been previously described.³ We newly established VAV1–STAP2-expressing Jurkat cells with a method similar to the previous one.³ SU9T01 cells inducibly expressing WT or G17V RHOA cDNA and mock-transduced cells were also established previously.

Jurkat cells and SU9T01 cells were cultured at 37 °C in RPMI-1640 Medium (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% fetal calf serum and 1% penicillin streptomycin. The 293T cells were cultured at 37 °C in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with 10% fetal calf serum and 1% penicillin streptomycin.

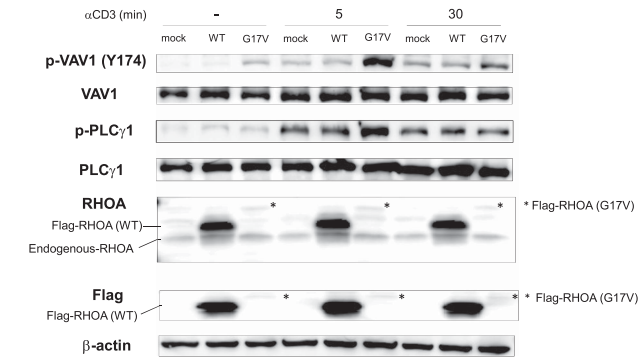


Figure 1. VAV1 activation by G17V RHOA in Jurkat cells. Jurkat^{mock}, Jurkat^{WTRHOA} and Jurkat^{G17V} cells were stimulated for 5 or 30 min with or without anti-CD3 antibody, followed by anti-mouse IgG antibody. Immunoblots of lysates were performed with antibodies to VAV1, phospho VAV1 (Tyr174), PLCγ1 and phospho PLCγ1. β-Actin served as loading control. p-PLCγ1, phospho PLCγ1; p-VAV1, phospho VAV1.

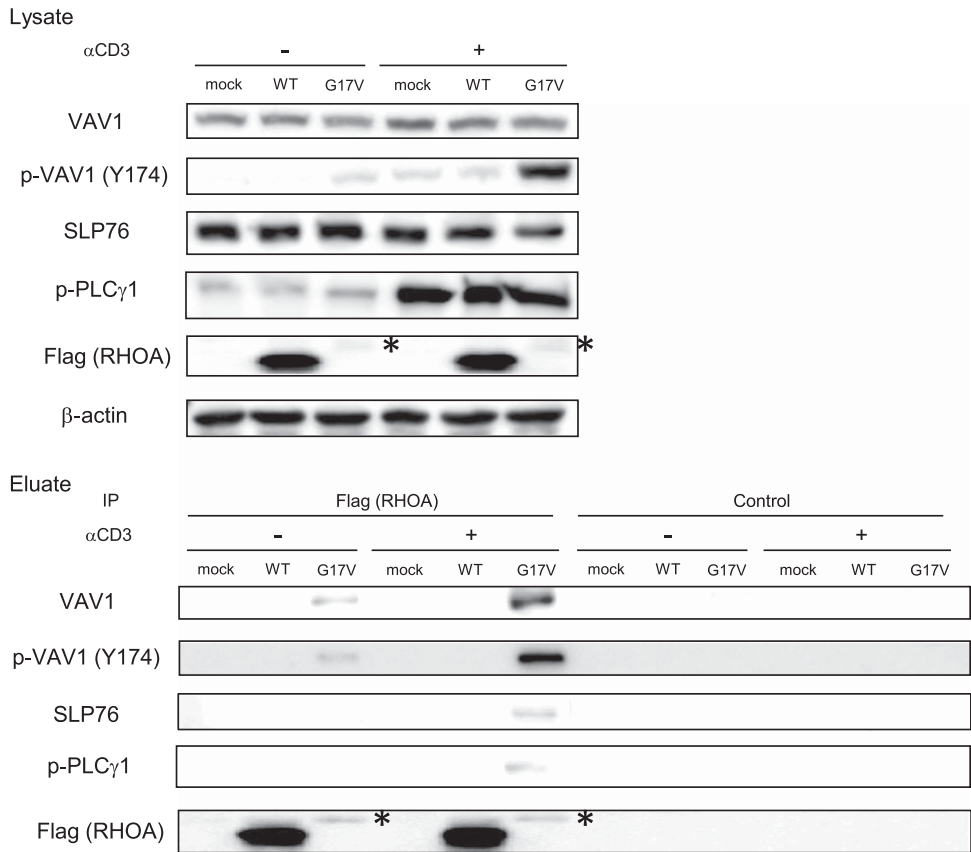


Figure 2. Binding of SLP 76 and phosphorylated PLCγ1 as well as VAV1 and phospho VAV1 to G17V RHOA in Jurkat cells. Jurkat^{mock}, Jurkat^{WTRHOA} and Jurkat^{G17V} cells were stimulated for 5 min with or without anti-CD3 antibody, followed by anti-mouse IgG antibody. Protein was immunoprecipitated from lysates using anti-Flag antibody and then immunoblotted with antibodies to Flag (RHOA), VAV1, phospho VAV1 (Tyr174), PLCγ1, phospho PLCγ1, and SLP76. β-Actin served as loading control. Asterisk (*) indicates G17V RHOA mutant. p-PLCγ1, phospho PLCγ1; p-VAV1, phospho VAV1.

Other experimental methods

DNA extractions, targeted sequencing of *RHOA*, *VAV1*, *PLCγ1*, *CD28*, *FYN* and *LCK* genes, antibodies, inhibitors, mRNA sequencing, plasmids, *in vitro* TCR stimulation, immunoprecipitation, mass spectrometry, NFAT reporter assay, interleukin-2 (IL-2) expression assay and immunohistochemistry are described in Supplementary Information.

RESULTS

Identification of G17V RHOA-specific binding proteins in Jurkat cells

To identify specific binding partners of G17V RHOA, we performed immunoprecipitation analysis in Jurkat cells overexpressing Flag-tagged WT RHOA (Jurkat^{WT-RHOA}) or the G17V RHOA mutant (Jurkat^{G17V}). Specifically, we used ANTI-FLAG Magnetic beads in either line, with or without TCR stimulation (Supplementary Figure 1). By targeted pull-down followed by mass spectrometry, we identified G17V RHOA-specific binding proteins: 44 proteins were identified specifically using G17V bait, both with and without TCR stimulation, 36 specifically without stimulation and 71 specifically with stimulation (Supplementary Table 1, rows 54–56). The corresponding database search results for the 44-protein subset showed the highest Mascot scores to be attributable to VAV family proteins, namely VAV1 and VAV3 (Supplementary Table 2, rows 77 and 78). Here, Mascot protein score is a surrogate for protein abundance. Notably, PLCγ1, an essential component of the TCR pathway, was detected among the G17V RHOA-specific binding proteins only with TCR stimulation (Supplementary Table 2, row 105).

Both tumor-associated G17V^{3–5} and engineered G17A RHOA mutants²⁶ lose nucleotide binding capacity, and both have been

shown to tightly bind various GEFs. Notably, in COS7 cells the G17A RHOA mutant interacts with active VAV1 mutants that lack the inhibitory N-terminal regions (Δ1–186) or C-terminal SH3 domains (Δ835–845).²⁵ Here, we performed western blot and immunoprecipitation analysis using Jurkat^{WT-RHOA} and Jurkat^{G17V} to validate mass spectrometry results reported above (Supplementary Figure 2). VAV1 was specifically coprecipitated with the G17V RHOA mutant but not with wild-type RHOA in the presence or absence of TCR stimulation, but binding efficiency was markedly augmented by TCR stimulation (Supplementary Figure 2). The result was consistent with the elevated Mascot score by mass spectrometry (Supplementary Table 2, row 78). In contrast, in similar assays we did not observe VAV3 binding to the G17V RHOA mutant (data not shown).

Various deletion mutants lacking the functional domains of VAV1 to determine the binding site for the G17V RHOA were made (Supplementary Figure 7a). A deletion mutant lacking the DH domain did not bind to the G17V RHOA, whereas other mutants as well as wild-type VAV1 did (Supplementary Figure 7b). These observations suggest that the DH domain is essential for binding to the G17V RHOA.

Interaction with G17V RHOA activates VAV1, enhancing its adaptor function

As described, VAV1 phosphorylation at Tyr174 is critical for its activation following TCR stimulation. Remarkably, we detected VAV1 Tyr174 phosphorylation in Jurkat^{G17V} cells, even in an unstimulated state, although at low levels, whereas Tyr174 phosphorylation was not seen in unstimulated Jurkat^{WT-RHOA} or mock-transduced cells (Figure 1 and Supplementary Figure 3). TCR stimulation enhanced VAV1 phosphorylation in both Jurkat^{WT-RHOA}

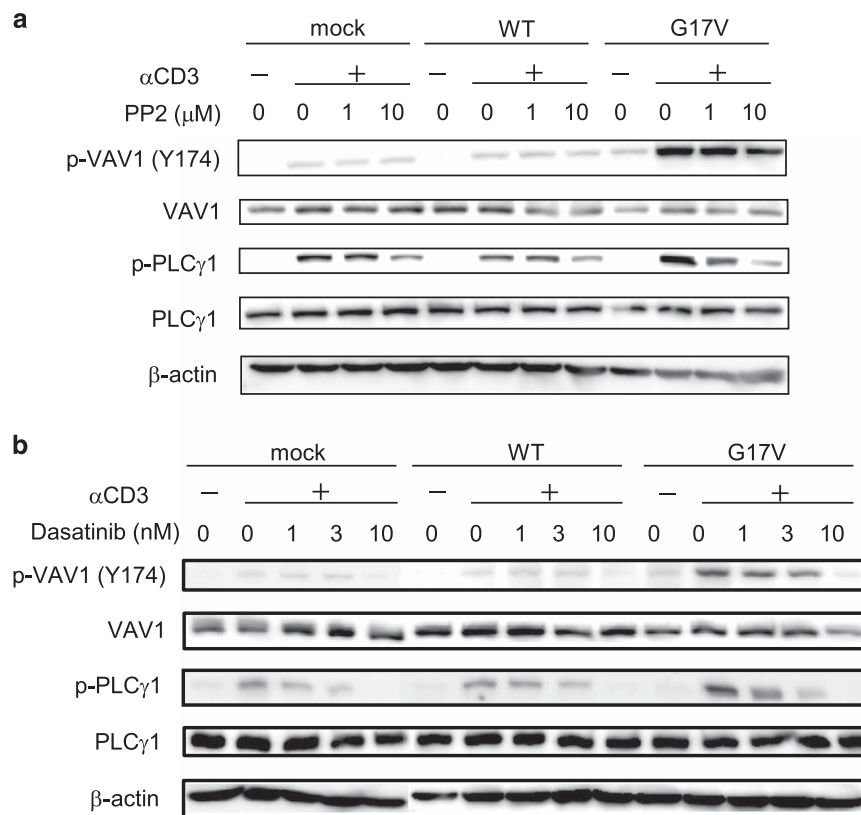


Figure 3. Effect of Src inhibitors on VAV1 activation by G17V RHOA following TCR stimulation. Jurkat^{mock}, Jurkat^{WT-RHOA} and Jurkat^{G17V} cells were stimulated with or without anti-CD3 antibody, followed by anti-mouse IgG antibody after (a) PP2 or (b) dasatinib treatment at indicated concentrations. Lysates were fractionated and immunoblotted with antibodies to VAV1, phospho VAV1 (Tyr174), PLCγ1 and phospho PLCγ1. β-Actin served as loading control. p-PLCγ1, phospho PLCγ1; p-VAV1, phospho VAV1.

and mock cells, but that effect was more pronounced in Jurkat^{G17V} cells (Figure 1 and Supplementary Figure 3). VAV1 mediates PLC γ 1 phosphorylation through facilitating assembly of a PLC γ 1–SLP76–LAT complex.¹⁹ As anticipated, PLC γ 1 phosphorylation was more striking in Jurkat^{G17V} than in mock-transduced or Jurkat^{WTRHOA} cells upon TCR stimulation (Figure 1 and Supplementary Figure 3).

SLP76, an adaptor molecule, and phosphorylated PLC γ 1 as well as total and phosphorylated VAV1 were also co-immunoprecipitated with G17V but not with wild-type RHOA (Figure 2). These observations suggest that G17V RHOA–VAV1 interaction enhanced the adaptor function of VAV1 to facilitate formation of a TCR signaling complex.

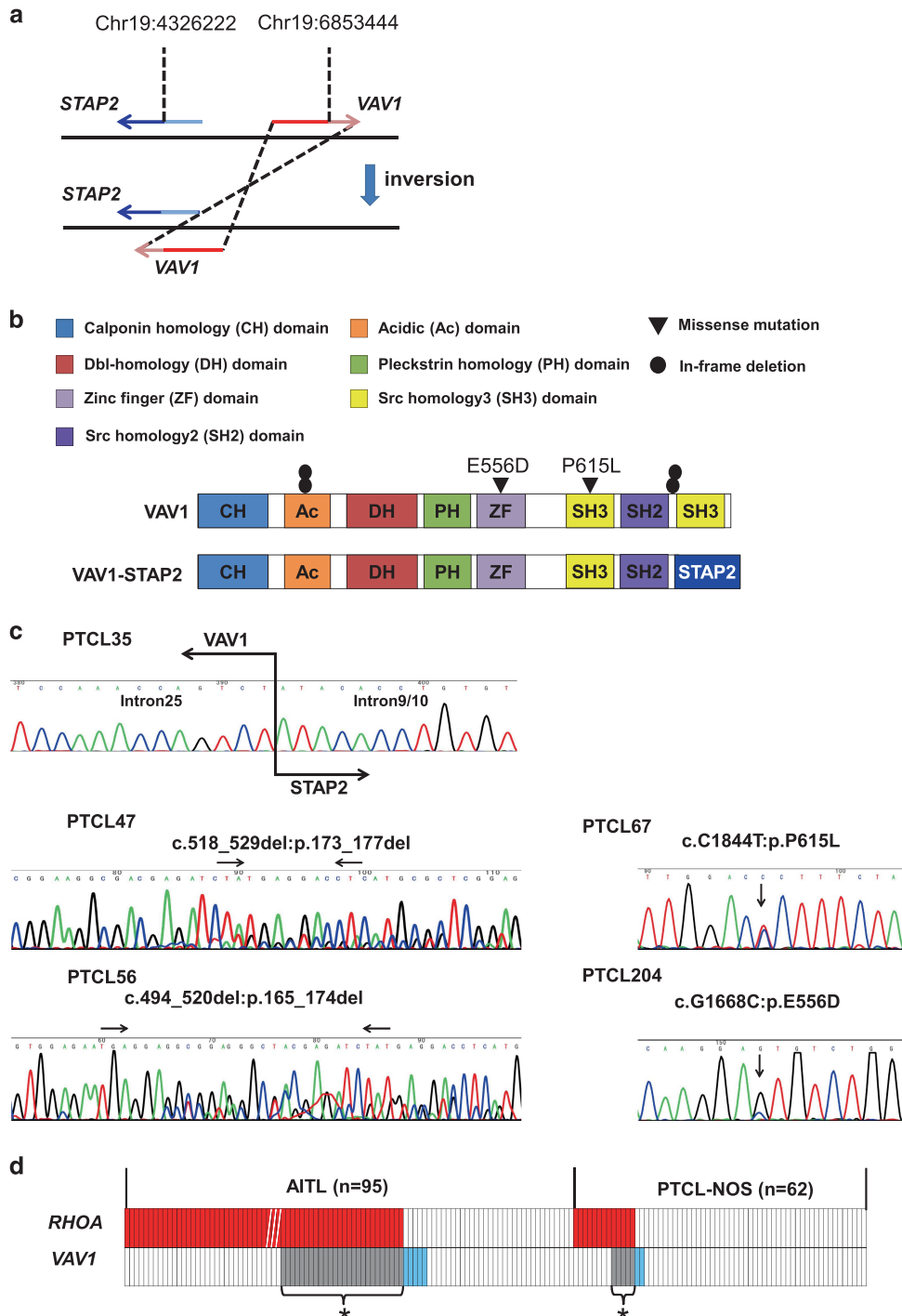


Figure 4. Identification of VAV1 mutations in human AITL and PTCL-NOS. **(a)** Schematic diagram of VAV1–STAP2 fusion genes. **(b)** Structure of VAV1 mutations and VAV1 functional domains. **(c)** Confirmation of VAV1 mutations by Sanger sequencing. Arrows indicate where mutations occur. **(d)** Mutation profile of *RHOA* and *VAV1* mutations. Three slushed samples had *RHOA* mutations other than typical c.G50T mutations. In PTCL 216, c.50_51GA>TC mutations resulted in p.Gly17Val alternation; in PTCL 223, c.50G>A mutation resulted in p.Gly17Glu alternation; and in PTCL 198, c.49_50GG>TT mutations resulted in p.Gly17Leu alternation. *, targeted sequencing for *VAV1* was not performed.

Src kinases reportedly mediate TCR signaling-dependent VAV1 phosphorylation. PP2, a pan-Src inhibitor, dose-dependently inhibited VAV1 phosphorylation (Figure 3a). In addition, some Src kinases including LCK and FYN are known targets of dasatinib, a drug used to treat BCR-ABL-positive leukemias. Thus, we asked whether dasatinib would inhibit VAV1 phosphorylation. As expected, dasatinib dose-dependently inhibited VAV1 phosphorylation, almost completely at 10 nM, in both Jurkat^{G7V} and Jurkat^{WT} cells (Figure 3b). PLCγ1 phosphorylation was also inhibited by PP2 and more efficiently by dasatinib (Figures 3a and b).

We also observed enhanced phosphorylation of VAV1 and PLCγ1 following induction of G17V RHOA expression in SU9T01 cells, an adult T-cell leukemia/lymphoma (ATLL) line, whereas induction of WT RHOA had no effect (Supplementary Figure 4). Furthermore, transient transduction of 293T cells with VAV1 cDNA plus either WT or G17V RHOA revealed greater VAV1 phosphorylation in the presence of G17V RHOA (Supplementary Figure 5). VAV1 and G17V RHOA also co-immunoprecipitated in 293T cells (Supplementary Figure 6). These data overall suggest that VAV1 activation in the presence of the G17V RHOA mutant occurs in a variety of cell types.

Activating VAV1 mutations occur in human AITL and PTCL-NOS VAV1 translocations and missense/deletion mutations have been identified in T-cell lymphomas.^{27–29} Here, RNA sequencing of 9 peripheral T-cell lymphoma (PTCL) samples (6 AITL and 3 PTCL-not otherwise specified (NOS)) identified a VAV1–STAP2 fusion gene in an AITL sample lacking the RHOA mutation (PTCL35, Figures 4a–c and Supplementary Tables 3 and 4). Targeted deep sequencing of VAV1 in 126 PTCL samples (69 AITL and 57 PTCL-NOS) identified two in-frame deletion mutations in an acidic region (c. C518_529del, p. 173_177del in PTCL47; c.C494_520del, p.165_174del in PTCL56) and two in-frame deletion mutations in C-terminal site of an SH2 domain (c.2333_2352del, p.778_786del²⁹ in PTCL181; c.2303_2329del, p.768_777del in PTCL213) in four AITL samples and two missense mutations in the zinc-finger domain and the SH3-SH2-SH3 module, respectively (c.G1668C, p. Glu556Asp in PTCL204; c.C1844T, p.Pro615Leu in PTCL67), in one PTCL-NOS and one AITL sample (Figures 4b and c and Supplementary Table 5). In total, seven VAV1 alterations including mutations of varying types and a fusion were found in 85 RHOA mutation-negative samples (8.2%), whereas none of the 41 RHOA mutation-positive PTCL samples exhibited VAV1 mutations (Figure 4d). In addition, we examined mutations in PLCG1, CD28, FYN and LCK for 49 samples. Six PLCG1 mutations, two CD28 mutations and one FYN mutation were detected, whereas LCK mutations were not (Supplementary Figure 8). Although the sample number was too small to elicit proper conclusion, both RHOA and VAV1 mutations were not exclusive with these mutations.

Analysis of Jurkat cells inducibly overexpressing VAV1–STAP2 (Jurkat^{VAV1–STAP2}) showed robust phosphorylation of VAV1–STAP2 at Tyr174, even in TCR-unstimulated conditions (Figure 5a and Supplementary Figure 9). VAV1 phosphorylation was faintly detected in cells overexpressing WT VAV1 (Jurkat^{WT/VAV1}) but not in mock-transduced (Jurkat^{mock}) cells when the TCR was unstimulated (Figure 5a and Supplementary Figure 9). TCR stimulation enhanced phosphorylation of both VAV1–STAP2 and WT VAV1, but VAV1–STAP2 was more highly phosphorylated than WT VAV1 (Figure 5a and Supplementary Figure 9). Unlike G17V RHOA expression, VAV1–STAP2 expression enhanced PLCγ1 phosphorylation, even without TCR stimulation (Figure 5a). These observations suggest that VAV1–STAP2 may autonomously activate TCR signaling without antigen stimulation. PLCγ1 phosphorylation was more striking in Jurkat^{VAV1–STAP2} than in mock-transduced or Jurkat^{WT/VAV1} cells upon TCR stimulation. Phosphorylation of VAV1–STAP2 and WT VAV1 was dose-

dependently inhibited by either PP2 or dasatinib treatment (Figures 5b and c).

Enhanced NFAT activity upon TCR stimulation by G17V RHOA and VAV1 mutants

NFAT transcription factors are essential for T-cell activation and differentiation, and TCR stimulation activates NFATs through Ca²⁺ mobilization.³⁰ VAV1 mediates Ca²⁺ mobilization mainly through PLCγ1 activation.¹⁹

To identify potential function of G17V RHOA in NFAT activity, we transiently transfected Jurkat cells with a reporter vector containing an NFAT response element plus WT or G17V RHOA cDNAs in the presence or absence of TCR stimulation. Expression of G17V RHOA augmented NFAT activity upon TCR stimulation, whereas that of the WT construct did not (Figure 6a). Dasatinib dose-dependently inhibited NFAT activity in Jurkat cells transduced with either WT or G17V RHOA (Figure 6b).

We also assessed NFAT activity in Jurkat cells transiently transduced with the NFAT response element reporter plus WT VAV1 or VAV1–STAP2, VAV1 p.173_177del, p.165_174del and p. Pro615Leu mutant cDNAs with or without TCR stimulation. In unstimulated conditions, the VAV1–STAP2 construct promoted induced NFAT activity in Jurkat cells as the previously reported fusions, VAV1–MYO1F and VAV1–S100A7²⁹ did, whereas the other mutants and WT VAV1 did not (Supplementary Figures 10a and

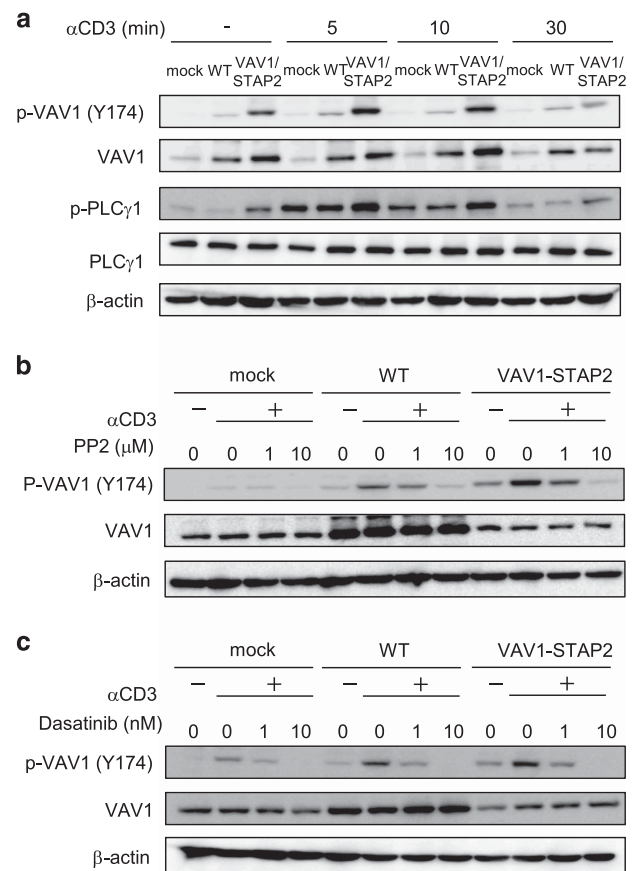


Figure 5. Effect of Src inhibitors on VAV1–STAP2 activation. (a–c) Jurkat^{mock}, Jurkat^{WT/VAV1} and Jurkat^{VAV1–STAP2} cells were stimulated with or without anti-CD3 antibody followed by anti-mouse IgG antibody. Cells were treated with (b) PP2 or (c) dasatinib at indicated concentrations. Lysates were fractionated and immunoblotted with antibodies to VAV1, phospho VAV1, and PLCγ1. β-Actin served as loading control. p-PLCγ1, phospho PLCγ1; p-VAV1, phospho VAV1.

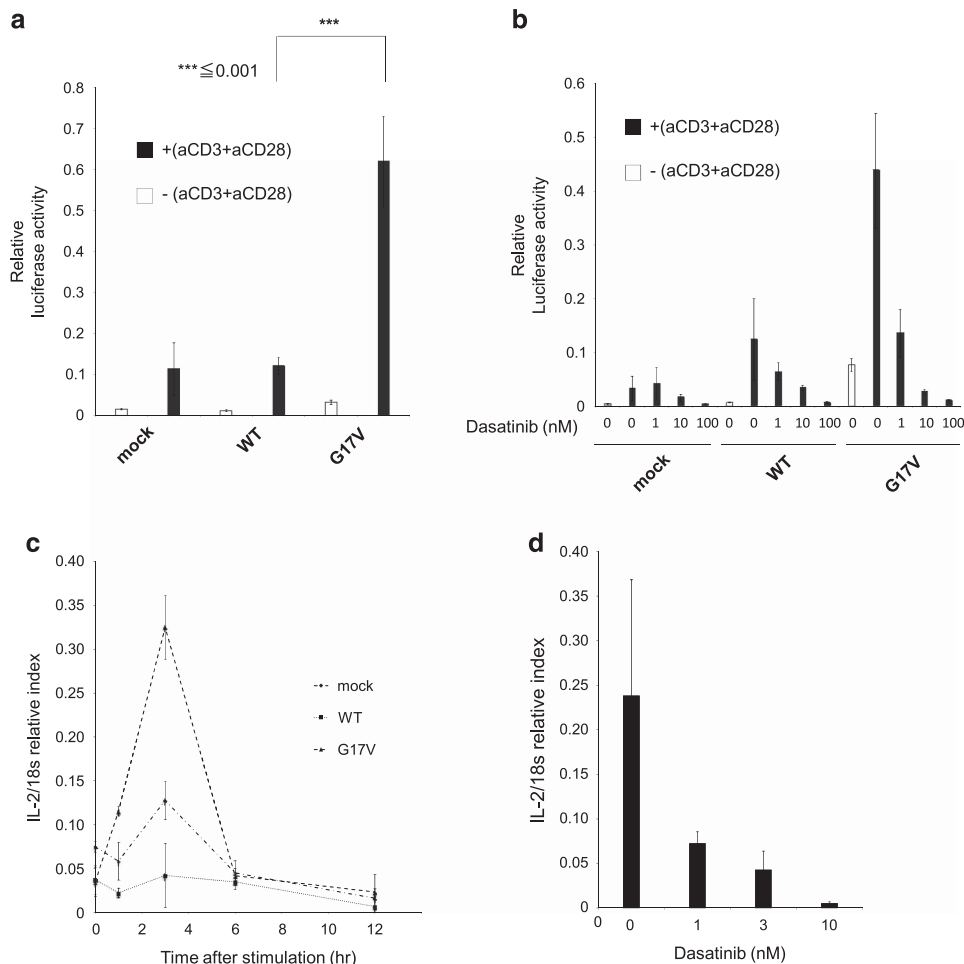


Figure 6. Effect of G17V RHOA on NFAT activity or IL-2 expression in Jurkat cells. **(a, b)** Jurkat cells were transiently transfected with a reporter containing an NFAT response element (NFAT-RE) together with WT or G17V RHOA mutant cDNAs in the presence or absence of Dynabeads Human T-activator CD3/CD28. **(a)** NFAT activity in indicated samples. **(b)** Effect of dasatinib on NFAT activity. The mean \pm s.d. from triplicate samples is shown. **(c, d)** Jurkat^{mock}, Jurkat^{WT RHOA} and Jurkat^{G17V} cells were stimulated in the presence or absence of Dynabeads Human T-activator CD3/CD28. **(c)** IL-2 gene expression based on real-time PCR. **(d)** Effect of dasatinib treatment on IL-2 expression in Jurkat^{G17V} cells. Cells were harvested at 3 h. The mean \pm s.d. from triplicate samples is shown.

11). Under TCR stimulation, NFAT activity was enhanced by transduction of WT VAV1 relative to mock control cells, whereas all VAV1 mutants had a greater enhancement effect than did WT VAV1 (Supplementary Figure 10a). Dasatinib treatment abrogated NFAT activity in Jurkat cells transduced with either WT or mutant VAV1 (Supplementary Figure 10b).

G17V RHOA and VAV1–STAP2 enhances IL-2 mRNA expression
IL-2 is a pivotal NFAT target gene.³⁰ We observed increased IL-2 mRNA expression following TCR stimulation of Jurkat^{mock}, peaking at 3 h and decreasing to slightly above basal levels by 6 h (Figure 6c). Such TCR stimulation-dependent IL-2 expression was markedly enhanced by G17V RHOA, whereas WT RHOA rather repressed TCR stimulation-dependent IL-2 mRNA expression (Figure 6c). Dasatinib almost completely suppressed IL-2 mRNA induction by TCR stimulation in Jurkat^{G17V} cells at doses that suppressed VAV1 phosphorylation (Figure 6d).

We also examined IL-2 expression in Jurkat^{VAV1–STAP2}, Jurkat^{WT VAV1} and Jurkat^{mock} cells in the presence or absence of TCR stimulation. IL-2 expression under TCR stimulation was higher in Jurkat^{VAV1–STAP2} than in Jurkat^{WT VAV1} or Jurkat mock control cells (Supplementary Figure 12a). IL-2 induction by TCR stimulation in

Jurkat^{VAV1–STAP2} was dose-dependently inhibited by dasatinib treatment (Supplementary Figure 12b).

Enrichment of cytokine- or chemokine-related signaling by G17V RHOA expression

We next performed mRNA sequencing analysis to examine effects of G17V RHOA on gene expression using RNA prepared from Jurkat cells inducibly expressing WT RHOA or G17V RHOA, or mock transduced, after CD3 and CD28 stimulation. Gene Set Enrichment Analysis demonstrated that cytokine–cytokine receptor interactions, chemokine signaling and TCR signaling pathways were significantly enriched at a false discovery rate q -value of < 0.25 for cells expressing WT RHOA or for mock-transduced cells (Figure 7, Supplementary Figure 13 and Supplementary Table 6).

AITL samples harboring *RHOA* or *VAV1* mutations show enhanced VAV1 Tyr174 phosphorylation

To determine whether VAV1 activation occurs in AITL tumor cells, we stained 26 human lymphoma samples using an antibody to VAV1 phosphorylated at Tyr174 (p-VAV1). In 12 AITL samples, all 8 samples with *RHOA* or *VAV1* mutations were p-VAV1-positive,

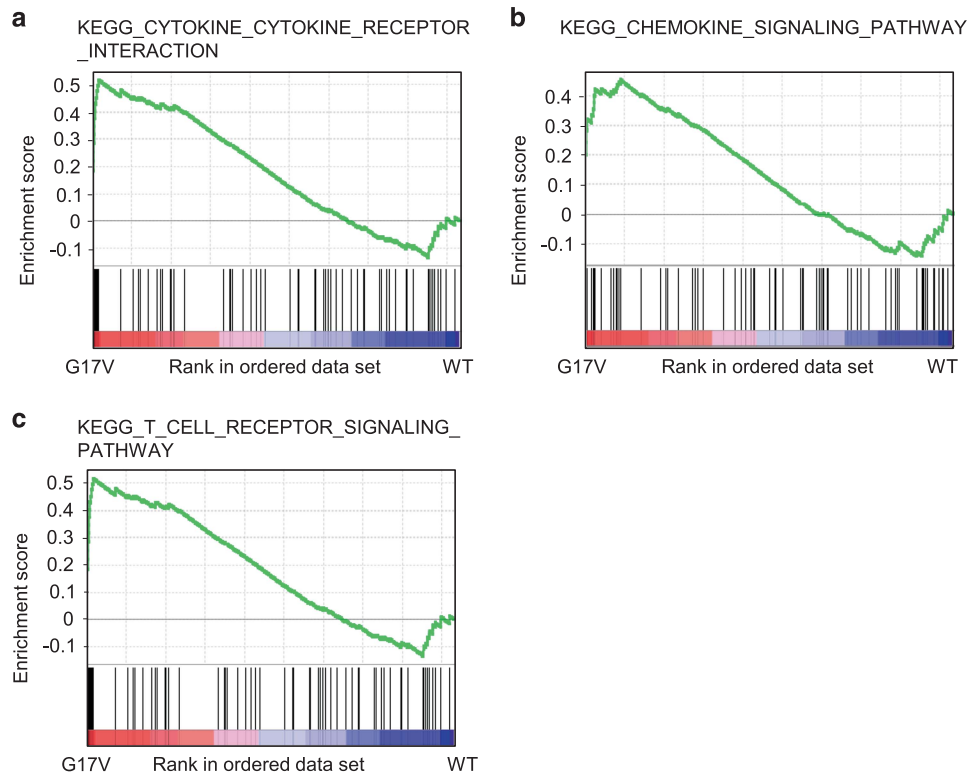


Figure 7. Transcriptome analysis of Jurkat^{G17V} and Jurkat^{WTRHOA} cells. Gene set enrichment analysis (GSEA) for Jurkat cells inducibly overexpressing WT or G17V RHOA or mock-transfected cells ($n = 2$ each). Representative differentially enriched pathways include (a) cytokine–cytokine receptor interactions, (b) chemokine signaling or (c) TCR signaling and refer to KEGG gene sets (C2) shown.

whereas 4 samples that did not exhibit mutations in either gene were unstained ($P < 0.05$) (Supplementary Table 7). Nine samples from patients with hematologic malignancies other than PTCL were negative for anti-p-VAV1 antibody. To determine the identities of anti-p-VAV1 antibody-positive cells, we performed double fluorescence staining in 11 PTCL samples exhibiting *RHOA* or *VAV1* mutations. The p-VAV1-positive cells costained with antibody to programmed cell death 1 (PDCD1), a marker of AITL tumor cells in 9 of 11 PTCL samples with *RHOA* or *VAV1* mutations (Figure 8 and Supplementary Table 7).

DISCUSSION

In AITL, the G17V *RHOA* mutation is among the most frequent gene alterations, whereas *VAV1* mutations are seen at much lower frequencies. All of these alterations if examined activate VAV1 protein through promoting tyrosine phosphorylation and enhance TCR signaling. The G17V *RHOA* and *VAV1* mutations were mutually exclusive in our cohort, though *VAV1* fusions were examined only in a part of samples.

Nonetheless, the physiologic functions of RHOA are presumably abrogated in the G17V mutant, as the protein cannot be converted to the active GTP-bound form. VAV1 mediates TCR signaling as both a GEF to activate small GTPases and as an adaptor to facilitate formation or function of the TCR signaling complex. The results reported here strongly indicate that G17V *RHOA* modulates VAV1 function as an adaptor (Supplementary Figure 14).

VAV1 mutations seen in AITL also enhance TCR signaling. Recent studies demonstrated that genes encoding members participating in TCR signaling are frequently mutated in AITL and related T-cell lymphomas.²⁸ Among genes encoding TCR signaling molecules, *PLCG1* (14.1%),²⁸ *CD28* (9.4–11.3%),^{28,31,32} *VAV1* (4.7%),^{28,29} and *FYN*

(2.9–3.5%)^{4,28} are most frequently mutated. *ITK*,^{27,33} *VAV1*^(refs. 27,29) and *CD28*^(ref. 34) loci are also involved in recurrent chromosomal translocations. This evidence combined with our functional analysis of G17V *RHOA* suggest that enhanced TCR signaling induces development of T-cell neoplasms, comparable to the idea that enhanced BCR signaling due to mutations is a critical factor in development of B-cell neoplasms.^{35,36}

In terms of gene expression, AITL tumor cells exhibit profiles comparable to follicular helper T cells,² and the transcription factors NFAT1 and NFAT2 are essential for follicular helper T-cell development.³⁷ Activation of NFAT by G17V *RHOA* may promote AITL tumor cell phenotypes. In addition, deregulated expression of ICOS, a signaling factor critical for follicular helper T-cell differentiation,³⁸ reportedly results in development of AITL-like disease in *Roquin*^{san} heterozygous mice.³⁹

Several reports show that cyclosporine A, which blocks TCR signaling by inhibiting calcineurin,⁴⁰ is effective to treat some AITL patients.^{41,42} Dasatinib, which inhibits the tyrosine kinase ABL in BCR-ABL-positive chronic myelogenous leukemia and acute lymphoblastic leukemia,⁴³ also inhibits other cytoplasmic and receptor tyrosine kinases.⁴⁴ We found that aberrant VAV1 activation and subsequent signaling by G17V *RHOA* and *VAV1* mutations are efficiently inhibited by dasatinib treatment. It is now important to conduct clinical studies to evaluate effects of dasatinib in AITL patients. G17V *RHOA* and *VAV1* mutations were seen in AITL samples positive for phospho-VAV1 at a statistically higher frequency. Thus, detection of phospho-VAV1 by immunostaining may serve as a surrogate biomarker for dasatinib sensitivity.

Recent genetic studies identified *RHOA* mutations in lymphoid malignancies other than AITL, such as Burkitt's lymphoma⁴⁵ and ATLL,⁴⁶ as well as in diffuse-type gastric carcinoma, a nonhematologic malignancy.⁴⁷ These findings suggest that structural

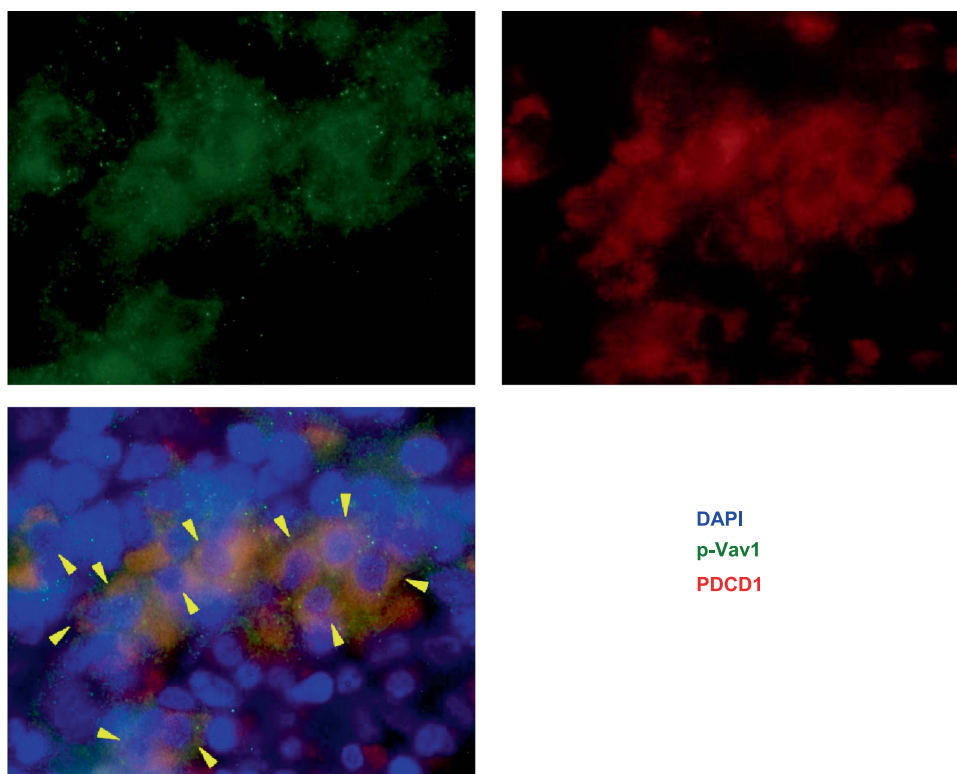


Figure 8. Immunostaining of phosphorylated VAV1 in human AITL samples. Immunofluorescence staining of an AITL sample to detect phosphorylated VAV1. Phosphorylated VAV1 (Tyr174, green) or PDCD1 (red) plus diamidino-2-phenylindole (DAPI) counterstaining. The yellow arrows indicate costained cells in p-VAV1 and PDCD1. Original magnification: $\times 600$ for all panels.

alteration of RHOA by mutation is a common mechanism underlying development of some malignancies. Mutational hot spots, however, differ among diseases: p.Arg5Gln and p.Tyr42Cys are most frequent in Burkitt's lymphoma⁴⁵ and gastric carcinomas,⁴⁷ whereas p.Cys16Arg is most frequent in ATLL.⁴⁶ Some RHOA mutants upregulate classical RHOA signaling whereas others downregulate it, even in a single disease.^{46,48} These outcomes are complex but possibly understandable if these RHOA mutants mediate oncogenesis through mechanisms other than modification of classical RHOA signaling as is shown here in AITL. VAV1 mutations are found in T-cell malignancies other than AITL, such as ATLL,⁴⁹ PTCL-NOS²⁷ and anaplastic large cell lymphoma,²⁷ as well as in nonhematologic cancers such as those of stomach,⁴⁷ pancreas⁵⁰ and melanoma.⁵⁰ Thus, RHOA and VAV1 mutations are frequent events in several cancers, although downstream mechanisms of these mutations in malignancies other than AITL remain to be elucidated.

In summary, our results demonstrate that the G17V RHOA–VAV1 axis is activated in AITL. This finding contributes to our understanding of AITL mechanisms and to efforts to develop targeted therapies.

CONFLICT OF INTEREST

SC received research funding from Bristol-Meyar Squibb that markets dasatinib.

ACKNOWLEDGEMENTS

We thank N Obara, Y Yokoyama, N Kurita and Y Hasegawa for contributing to sample collection and banking. We also thank Y Sakashita and T Takahashi for technical assistance. We are grateful to Teresa Palomero for providing us with VAV1 fusion cDNAs and Naomichi Arima for SU9T01 cells. This work was supported by Grants-in-Aid for Scientific Research (KAKENHI) from the Ministry of Education, Culture, Sports,

Science and Technology of Japan (25112703, 15H01504 and 16H02660 to SC and JP16K15497 to MS-Y) and the Project for Cancer Research and Therapeutic Evolution (P-CREATE) from the Japan Agency for Medical Research and Development, AMED, to SC. Mass spectrometry was supported by NIH SIG 1S10OD016328 to P Gershon. This study was partly supported by TaNeDS program (Daiichi Sankyo).

AUTHOR CONTRIBUTIONS

M F, MS-Y and SC wrote the manuscript; MF, MS-Y, SN, DK, M K, ST, K F and TE contributed to functional analysis; P Gershon performed mass spectrometry; MM, KY and SO performed mRNA sequencing; MF, HM and KO performed immunostaining; KM, NN, KT, KI, K Fujimoto, TT and P Gaulard contributed to sample collection and vital discussion.

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