STAT3 mutations identified in human hematological neoplasms induce myeloid malignancies in a mouse bone marrow transplantation model


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STAT3 mutations identified in human hematological neoplasms induce myeloid malignancies in a mouse bone marrow transplantation mode

Running title: STAT3 mutations induce myeloid malignancies in mice.


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Key words: STAT3, lymphoid, leukemia, lymphoma, mouse model
Abstract

STAT3 protein phosphorylation is a frequent event in various hematological malignancies and solid tumors. Acquired STAT3 mutations have been recently identified in 40% of patients with T large granular lymphocytic leukemia, a rare T-cell disorder. In this study, we investigated the mutational status of STAT3 in a large series of patients with lymphoid and myeloid diseases. STAT3 mutations were identified in 1.6% (4/258) of patients with T-cell neoplasms, in 2.5% (2/79) of patients with diffuse large B-cell lymphoma but in no other B-cell lymphoma patients (0/104) or patients with myeloid malignancies (0/96). Functional in vitro assays indicated that the STAT3Y640F mutation leads to a constitutive phosphorylation of the protein. STA21, a STAT3 small molecule inhibitor, inhibited the proliferation of two distinct STAT3 mutated cell lines. Using a mouse bone marrow transplantation assay, we observed that STAT3Y640F expression leads to the development of myeloproliferative neoplasms with expansion of either myeloid cells or megakaryocytes. Together, these data indicate that the STAT3Y640F mutation leads to constitutive activation of STAT3, induces malignant hematopoiesis in vivo and may represent a novel therapeutic target in some lymphoid malignancies.
Introduction

STAT transcription factors are key regulators of gene transcription activated by cytokine receptors and JAK signaling and are involved in numerous cellular processes including hematopoietic differentiation, proliferation and immune responses. Aberrant activation of STAT factors, including STAT3, is frequently observed in hematological malignancies and solid tumors. It is generally thought that aberrant STAT activation results from mutation or constitutive activation of upstream members of the signaling pathway, like in myeloproliferative neoplasms (MPN) in which JAK2 and MPL genes are frequently mutated activating several STAT factors including STAT3.\textsuperscript{1,2} Somatic mutations of STAT3 associated with a constitutive phosphorylation, dimerization and activation of STAT3 have been identified in 6\% of human inflammatory hepatocellular adenomas\textsuperscript{3} and may be a hallmark of T-cell large granular lymphocytic leukemia (TGLL).\textsuperscript{4} In a number of hematological malignancies, the mechanism of constitutive STAT3 activation remains unexplained.\textsuperscript{5}

Methods

Patients. Lymph node and peripheral blood samples from the patients were obtained with their informed consent and the approval of the local Research Ethics Committees (Centre Henri Becquerel, Pitié-Salpêtrière, Cochin, Bordeaux and Toulouse hospitals). Diagnoses were made by standard international criteria.

Polymerase chain reaction (PCR) and DNA sequencing. PCR primers (Online Supplementary Table S1) were designed to amplify and sequence 5 coding exons of STAT3 (3, 6, 17, 21, 22). Nucleotide sequences were compared to wild-type human genomic sequence present in the databases (genome.ucsc.edu). All observed STAT3 mutations were detected by bidirectional sequencing.

RNA seq. RNA-seq was performed on an Illumina HiSeq 2000 using paired-end sequencing of 150–250-bp inserts and 100-bp reads (Fasteris).

Constructs. Full-length WT and mutant Y640F STAT3 open reading frame were cloned from a pCMV6-XL4 vector into a MSCV240-IRES-GFP retroviral vector.\textsuperscript{3}

Cell culture. Ba/F3, YT1, FEPD and K562 cells were cultured in RPMI 1640 containing 10\% fetal bovine serum (FBS). For growth of Ba/F3, medium was supplemented with 10 ng/mL mouse interleukin 3 (IL-3). For infections, Ba/F3 were mixed with viral supernatants obtained following standard procedures, spininfected for 90 minutes at 1800 g, and returned to the incubator. 48 hours after infection, cells were sorted for GFP expression. For pharmacological treatment, cells were cultured in regular media with STA-21, a small STAT3 molecule inhibitor (25 μM), for 72 hours. The number of viable cells was assessed by counts every day using the trypan blue exclusion method.

Western blot (WB) analysis and immunoprecipitation. WB and immunoprecipitation were performed following standard procedures and using 20.10\(^6\) cells for each point. Eluates obtained after immunoprecipitation were analyzed by Western blot for STAT3 C20 (Santa Cruz) and phospho-STAT3 Tyr705 (Cell Signaling).
Flow cytometry. For phospho-specific flow cytometry, cells were fixed in methanol-free formaldehyde 1% for 10 min, washed with PBS, permeabilized with ice-cold 100% methanol for 30 min and saturated with 0.5% BSA overnight. Total white blood cells and single-cell suspensions from bone marrow and spleen were stained in toto in PBS supplemented with 2% FBS with fluorochrome-conjugated mouse antibodies (Online Supplementary Table S2) raised against specific hematopoietic lineages markers.

Animal experiments. Wild-type (WT) C57BL/6 donor mice were injected with 5-fluorouracile (5-FU) 5 days prior to bone marrow (BM) collection. On day 0, primary BM cells were obtained from femurs and tibiae, subjected to red blood cell lysis buffer and cultured overnight in RPMI 1640 supplemented with 10% FBS+IL-3, IL-6, and SCF (10ng/ml each). On day 1 and day 2, cells were mixed with identical viral supernatants and spininfected for 90 minutes at 1800 g. After the second spinfection, 1 x 10^6 cells were injected in the tail veins of lethally irradiated C57BL/6 recipients. After transplantation, animals were monitored daily for signs and symptoms of disease and monthly by blood counts. The percentages of GFP-positive cells at various time-points after transduction are indicated in Online Supplementary Figure S2. Transplantation into sub-lethally irradiated secondary syngeneic recipients was performed using a 1-to-1 mixture of bone marrow cells (1.10^6 cells) and splenocytes (1.10^6 cells) from three independent primary recipients mice.

Results

We identified by RNA-sequencing and confirmed by Sanger sequencing a STAT3 Y640F mutation in the hematopoietic NK/T cell line YT1 (Online Supplementary Figure S1A). We then investigated the mutational status of STAT3 in a large cohort of patients with myeloid or lymphoid disorders. Based on previously reported STAT3 mutations, we performed Sanger sequencing of 5 coding exons of STAT3 (exons: 3, 6, 17, 21, 22), including the exons encoding the Src homology 2 (SH2) domain. Among B cell neoplasms, STAT3 mutations were detected in 2.5% of patients with diffuse large B-cell lymphoma (2/79) (Table 1 and Online Supplementary Figure S1A). No STAT3 mutation was found in T-cell rich B-cell lymphoma (n=3), follicular lymphoma (n=60), mantle cell lymphoma (n=19), marginal zone lymphoma (n=12), or lymphocytic B lymphoma (n=9) (Table 1). We identified STAT3 mutations in 4 of 258 patients (1.6%) with T-cell neoplasms: two patients with a cutaneous CD30+ ALK-negative anaplastic large cell lymphoma (cALCL ALK- CD30+), one with a peripheral T-cell lymphoma not otherwise specified and one with a gamma-delta T-cell lymphoma (Table 1 and Online Supplementary Figure S1A). A clinical summary of STAT3 mutated patients is reported in Online Supplementary Table S3. For one of the cALCL ALK- CD30+ patient, the blood cells in which no tumoral cells were detected did not present the mutation (Online Supplementary Figure S1A), supporting that the STAT3 mutation was acquired in the malignant cells. A STAT3 G618R mutation was also identified in FEPD, an ALK- ALCL cell line (Online Supplementary Figure S1A). All the mutations identified in patient cells were heterozygous and were located in the SH2 domain (Y640F, Y657ins, E616del (x2), D661ins, D661Y). The position D661 was recurrently affected in accordance with the recent report by Koskela et al. and the missense mutation located at E616 had also been previously described in one patient with DLBCL. No mutation was observed in myeloid malignancies, including wild-type JAK2 primary myelofibrosis (PMF) (n=7), wild-type JAK2 essential thrombocytemia (ET) (n=40) and chronic myelomonocytic leukemia (n=49). These results were
consistent with a previous study that did not detect mutation of the JAK/STAT pathway (JAK1, JAK3, TYK2, STAT5A, and STAT5B) in wild-type JAK2 ET and PMF patients.\(^7\)

To investigate the functional consequences of STAT3 mutations, we then analyzed STAT3 activation and phosphorylation in the YT1 and FEPD cell lines. Both lines showed constitutive STAT3 phosphorylation by flow cytometry (Figure 1A) and western blotting (Figure 1B). Importantly, treatment of YT1 and FEPD with STA-21, a STAT3 small molecule inhibitor, resulted in a proliferation arrest of both STAT3 mutated cell lines, whereas the STAT3 wild-type K562 cell line still proliferated (Figure 1C). Of note, STA-21 treatment is significantly more effective in FEPD in which the STAT3 mutation is homozygous than in YT1 presenting an heterozygous STAT3 mutation (Figure 1C and Online Supplementary Figure S1A). These results suggest that STAT3 mutations induce a constitutive phosphorylation of STAT3 and participate in the proliferation of YT1 and FEPD cells.

Next, we transduced the IL3-dependent murine Ba/F3 cells with empty (control), wild-type (WT) STAT3 or STAT3 Y640F MSCV retroviruses. Western blotting confirmed that ectopic expression of STAT3 Y640F mutant induced constitutive phosphorylation of STAT3 compared to WT STAT3 and control cells (Figure 1D). Expression of STAT3 Y640F did not affect the cytokine dependency of the Ba/F3 cells and no difference in proliferation was observed when BA/F3 cells were cultured in the presence of IL4 or FLT3 ligand (Online Supplementary Figure S1B). These results indicate that although STAT3 Y640F induced constitutive phosphorylation of STAT3, this mutant is not sufficient to confer cytokine-independent growth to Ba/F3 cells.

To investigate the role of STAT3 mutations in vivo, we performed bone marrow transplantation (BMT) assays using bone marrow cells from 5-fluorouracil treated wild-type C57BL/6 transduced with STAT3 Y640F, STAT3 WT or empty retroviruses and injected to lethally irradiated syngenic recipients (Online Supplementary Figure S2). All the ten STAT3 Y640F recipients developed symptoms of hematological disease within one to three months and nine were sacrificed between one and seven months after BMT for analysis. STAT3 Y640F mice presented MPN-like diseases with slight hyperleukocytosis (except for one mouse) (Figure 2A), a trend toward anemia (Figure 2B) and a significant splenomegaly (p=0.04, Unpaired t-test with Welch’s correction) (Figure 2D). Two distinct phenotypes were observed. Three mice developed a thrombocytosis disorder characterized by a progressive increase of the platelets counts (Figure 2C) associated with a severe anemia (Figure 2B). One animal, which initially presented thrombocytosis 4 months after BMT, showed platelet counts returning to normal with a concomitant decrease in the number of GFP positive cells (9 months follow-up: Online Supplementary Figure S1C). Flow cytometry analysis of this subgroup indicated an alteration of the erythroid lineage maturation with a reduction in CD71+Ter119+ erythroblasts and a marked increase in CD41+CD42+ bone marrow megakaryocytes (Figure 2E). Seven mice presented a myeloid disease, with a transient hyperleukocytosis (except for one animal, which succumbed early after BMT with a high hyperleukocytosis) (Figure 2A), a progressive anemia (Figure 2B) and trend toward thrombocytopenia (Figure 2C). Flow cytometry analysis indicated an abnormal Mac+Gr1+cKit+population infiltrating the bone marrow (Figure 2E), spleen and blood (data not shown) and defects in erythroid lineage maturation with accumulation of CD71+Ter119Iow cells in the bone marrow (Figure 2E). No symptoms of disease or hematological abnormalities were detected in empty MSCV or STAT3 WT recipients within this time lapse (Figure 2A-E). Also, no T-cell differentiation alteration was detected in either group. Of importance, the MPN-like diseases observed in primary STAT3
Y640F recipients, either thrombocytosis or myeloid diseases, could not be transplanted to secondary recipients with over 6 months of follow-up (Online Supplementary Figure S2). Together, these data indicate that STAT3 Y640F induced an MPN-like disease in a bone marrow transplantation model.

Discussion

Here, we reported that STAT3 mutations present a rare incidence in patients with lymphoid and myeloid diseases in general. We detected STAT3 mutations in a rare subset of DLBCL patients (2.5%), which is consistent with recent analysis of DLBCL using high throughput genome sequencing and in few patients with T-cell lymphoma. During the course of this work, STAT3 mutations were reported as frequent event in TGLL and in chronic lymphoproliferative disorders of NK cells (CLPD-NK). All observed STAT3 mutations were located in exon 21, encoding the SH2 domain and resulted in phosphorylation, dimerization and activation of the protein. Of note, no STAT3 mutation was observed in our cases of TGLL, but this may result from the small number of analyzed patients.

Our data also support the hypothesis that STAT3 mutations participate in the tumorigenesis. Indeed, we demonstrate that the STAT3 Y640F mutation leads to constitutive phosphorylation of STAT3 and efficiently induces MPN with either myeloid or thrombocytosis features in a murine retroviral transduction-bone marrow transplantation model. Of note, these results are consistent with previous data showing the importance of STAT3 for granulopoiesis and megakaryopoiesis. Indeed, STAT3 has been described as an essential component of G-CSF-driven cell proliferation and granulopoiesis. STAT3 is also important for both normal megakaryocyte development and in some murine models of ET. Of note, mice homozygous for a truncation mutation in gp130, deleting all STAT3 binding sites, show altered platelet production. In addition, the expansion in the number of immature hematopoietic progenitor, thrombocytosis, and splenomegaly observed in a murine model of constitutive activation of gp130 can be rescued by genetic ablation of STAT3. Although these data strongly suggest that STAT3 has a cell-autonomous contribution to normal and malignant megakaryocyte development, we cannot exclude that aberrant release of cytokines participate in the thrombocytosis observed in some mutant STAT3 recipients.

In this bone marrow transplantation setting, we observed the development of myeloid diseases without T or NK cell pathologies. This discrepancy has been observed in other instances. For example, gain-of-function JAK2 alleles detected in patients with Down's syndrome acute lymphoblastic leukemia induced myeloproliferative disorders using a similar bone marrow transplant setting. Therefore, further studies are needed to elucidate the role of STAT3 mutations in the transformation of lymphoid lineages and will likely require expression of these STAT3 mutant specifically in lymphoid lineages or their co-expression with cooperating mutations. Identification of the surface receptor interacting with STAT3 may also provide a better understanding of the constitutive activation of STAT3 observed in these human hematopoietic malignancies. Finally, these data may also be clinically relevant. Indeed, we observed that the proliferation of T-cell lymphoma cell lines presenting a STAT3 mutation is inhibited by the STAT3 inhibitor STA-21. As STAT3 mutations affect 40% of TGLL patients and 30% of CLPD-NK patients, it will be interesting to investigate further the efficacy of STAT3 inhibitors as a novel targeted therapy in these specific and rare pathologies.
Acknowledgements
We thank Dr. Patrick Gonin and Laure Touchard from Institut Gustave Roussy animal facilities and Philippe Rameau from the Institut Gustave Roussy Flow Cytometry Core Facility. This work was funded by grants from INSERM, Institut National du Cancer (INCa), Ligue Nationale Contre le Cancer (LNCC), Association de Recherche contre le Cancer (ARC) and Fondation Gustave Roussy. Lucile Couronné was supported by a PhD fellowship from INSERM. Laurianne Scourzic was supported by a PhD fellowship from INCA.

Authorship and disclosures
LC, LS, CP and VDV performed the laboratory work; YD performed the bioinformatics analysis; ES, WV, JPM, FD, MHS, PG, LI, ED, HMB, FNF, MF, HT, and CB collected and provided patients samples; CP and JZR provided vital reagents, OAB and TM coordinated the research; LC, OAB and TM wrote the manuscript.
The authors report no potential conflicts of interest
References


### Table 1

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<tr>
<th>Diagnosis</th>
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<td><strong>B-cell neoplasms</strong></td>
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<td>DLBCL</td>
<td>79</td>
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<td>TCRBL</td>
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<td>FL</td>
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<td>0</td>
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<tr>
<td><strong>T-cell neoplasms</strong></td>
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<tr>
<td>cALCL ALK-</td>
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<td>2 (20%)</td>
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<tr>
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<tr>
<td>T-ALL/LBL</td>
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<tr>
<td>PMF Jak2 unmutated</td>
<td>7</td>
<td>0</td>
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<tr>
<td>ET Jak2 unmutated</td>
<td>40</td>
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<tr>
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<td>49</td>
<td>0</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>537</td>
<td>6 (1.1%)</td>
</tr>
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</table>

**Table 1:** STAT3 mutations in lymphoid and myeloid neoplasms.

DLBCL, diffuse large B cell lymphoma; TCRBL, T cell-rich B cell lymphoma; FL, follicular lymphoma; MCL, mantle cell lymphoma; MZL, marginal zone lymphoma; AITL, angioimmunoblastic T cell lymphoma; ALCL ALK+, anaplastic large cell lymphoma ALK positive; ALCL ALK-, anaplastic large cell lymphoma ALK negative; ATL, adult T cell lymphoma/leukemia (HTLV1+); TGLL, T cell large granular lymphocytic leukemia; TLBL, precursor T-lymphoblastic lymphoma; TPLL, T cell prolymphocytic leukemia; PMF, primary myelofibrosis; ET, essential thrombocytosis; CMML, chronic myelomonocytic leukemia.
**Figures Legends**

**Figure 1:**
A. STAT3 phosphorylation analysis by flow cytometry of YT1 and FEPD (STAT3 mutated cell lines) as compared to K562 (STAT3 WT cell line). Control represents the unstained cells. Analysis was performed in normal conditions and after serum starvation.
B. STAT3 phosphorylation analysis by Western Blotting of YT1 and FEPD (STAT3 mutated cell lines) as compared to K562 (STAT3 WT cell line).
C. Proliferation assay of YT1, FEPD (STAT3 mutated cell lines) and K562 (STAT3 WT cell line) after treatment with the STAT3 inhibitor STA-21 (25 μM) (left panel) as compared to DMSO treatment (middle panel). The right panel shows the relative cell counts at 72-hours of STA-21 treatment normalized to the number of DMSO-treated cells for each cell line.
D. STAT3 phosphorylation analysis by Western Blotting of transduced murine Ba/F3 cells with empty (-), wild-type (WT) STAT3 or STAT3 Y640F MSCV retroviruses.

**Figure 2:**
A. White blood cells (WBC) count in peripheral blood samples obtained from MSCV, STAT3 WT and STAT3 Y640F mice. The x-axis values represent the number of months after bone marrow transplantation.
B. Hemoglobin level in peripheral blood samples obtained from MSCV, STAT3 WT and STAT3 Y640F mice. The x-axis values represent the number of months after bone marrow transplantation.
C. Platelets count in peripheral blood samples obtained from MSCV, STAT3 WT and STAT3 Y640F mice. The x-axis values represent the number of months after bone marrow transplantation.
D. Spleen weight of MSCV, STAT3 WT and STAT3 Y640F mice.
E. Representative flow cytometrical analysis of the mature myeloid cells (left), erythroid (center) and megakaryocytic (right) lineages in the bone marrow of STAT3 Y640F mice with myeloid and thrombocytosis diseases, as compared to MSCV and STAT3 WT mice. The percentages of GFP-positive cells are indicated.
**A**

WBC

- MSCV
- STAT3 WT
- STAT3 Y640F

**B**

Hemoglobin

- MSCV
- STAT3 WT
- STAT3 Y640F

**C**

Platelets

- MSCV
- STAT3 WT
- STAT3 Y640F

**D**

Spleen weight (mg)

- MSCV
- WT
- Y640F

**E**

MSCV

- 7.4
- 57
- 15
- 2.4
- 2.6
- 0.8

STAT3 WT

- 9.2
- 50
- 11
- 4.1
- 2.1
- 1.1

STAT3 Y640F (Myeloid)

- 8.8
- 81
- 40
- 3.6
- 3
- 1.6

STAT3 Y640F (Thrombocytosis)

- 15
- 58
- 21
- 1.8
- 1.6
- 17

**Legend**

- Mac1
- Gr1
- CD71
- Ter119
- CD41
SUPPLEMENTAL DATA

**STAT3 mutations identified in human hematological neoplasms induce myeloid malignancies in a mouse bone marrow transplantation model.**

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**Supplemental data legends**

**Figure S1:** STAT3 mutations in patients and expression in cell lines and *in vivo* murine models.

A. *STAT3* sequences in tumor samples.

Vertical arrows indicate the sequence variations. Horizontal arrows indicate the origin of the indel mutations.
B. Proliferation assay of transduced murine BAF3 cells with empty, wild-type (WT), STAT3 or STAT3 Y640F MSCV retroviruses in presence of IL3, IL4 or FLT3 ligand. Control corresponds with the condition without cytokines.

C. White blood cells (WBC) count, hemoglobin level, platelets count and percentage of GFP-positive cells follow up of the individual 395 (STAT3 Y640F mice with thrombocytosis disease). The x-axis values represent the delay in months after bone marrow transplantation.

**Figure S2:** Percentages of GFP-positive cells at different time-points after transduction

A. Transduction efficiencies in the bone marrow cells right after the second spinfection and just before the injection into the primary recipients.

B. Representative percentages of GFP in the blood leukocytes of primary recipients at 1 month post-transplantation.

C. Percentage of GFP-positive cells in the bone marrow and spleen cells from three independent STAT3Y640F primary recipients at the time of transplantation into secondary recipients.

**Table S1:** List of primers used for STAT3 PCR and sequencing

**Table S2:** Antibodies combinations used for flow cytometry

**Table S3:** Clinical data of STAT3 mutated patients
Figure S2

A

MSCV  STAT3 WT  STAT3 Y640F

B

MSCV  STAT3 WT  STAT3 Y640F

C

BM

#1  #2  #3

STAT3 Y640F

Spleen

SSC

15.5  11.4  17.2

59.9  45.4  78.8

4.77  37.7  53.7

25.4  28.7  46.1
Table S1: List of primers used for STAT3 PCR and sequencing

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<td>STAT3exon6F</td>
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<td>STAT3exon17R</td>
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<td>Antibody</td>
<td>Clone</td>
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<tr>
<td>CD11b (Mac)</td>
<td>M1/70</td>
</tr>
<tr>
<td>Ly-6G (Gr-1)</td>
<td>RB6-8C5</td>
</tr>
<tr>
<td>CD41</td>
<td>MWReg30</td>
</tr>
<tr>
<td>CD42b (GPIbα)</td>
<td>Xia.G5</td>
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<td>CD71</td>
<td>R17 217.1.4</td>
</tr>
<tr>
<td>Ter119</td>
<td>TER-119</td>
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</tbody>
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PE: phycoerythrin, APC: allophycocyanin, PE-Cy7: phycoerythrin-cyanine 7
<table>
<thead>
<tr>
<th>Table S3: Clinical data of STAT3 mutated patients</th>
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<tr>
<td><strong>PTCL, NOS Y640F</strong> 60-year-old man</td>
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<tr>
<td><strong>Gamma-delta T lymphoma Y657ins</strong> 38-year-old man</td>
</tr>
<tr>
<td><strong>DLBCL E616del #1</strong> 62-year-old man</td>
</tr>
<tr>
<td><strong>DLBCL E616del #2</strong> 56-year-old man</td>
</tr>
<tr>
<td><strong>cALCL D661ins</strong> 72-year-old man</td>
</tr>
<tr>
<td><strong>cALCL D661Y</strong> 82-year-old woman</td>
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