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JAK3 mutants transform hematopoietic cells through JAK1 activation causing T-cell acute lymphoblastic leukemia in a bone marrow transplant mouse model.

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KEY POINTS

- JAK3 pseudokinase mutants require JAK1 for their transforming potential.
- JAK3 mutants cause T-ALL in a mouse bone marrow transplant model and respond to tofacitinib, a JAK3 selective inhibitor.

ABSTRACT

JAK3 is a tyrosine kinase that associates with the common gamma chain of cytokine receptors and is recurrently mutated in T-cell acute lymphoblastic leukemia (T-ALL). We tested the transforming properties of JAK3 pseudokinase and kinase domain mutants using *in vitro* and *in vivo* assays. Most, but not all, JAK3 mutants transformed cytokine dependent Ba/F3 or MOHITO cell lines to cytokine independent proliferation. JAK3 pseudokinase mutants were dependent on Jak1 kinase activity for cellular transformation, whilst the JAK3 kinase domain mutant could transform cells in a Jak1 kinase independent manner. Reconstitution of the IL7 receptor signaling complex in 293T cells showed that JAK3 mutants required receptor binding to mediate downstream STAT5 phosphorylation. Mice transplanted with bone marrow progenitor cells expressing JAK3 mutants developed a long latency transplantable T-ALL like disease, characterized by an accumulation of immature CD8 positive T-cells. *In vivo* treatment of leukemic mice with the JAK3 selective inhibitor tofacitinib reduced the white blood cell count and caused leukemic cell apoptosis. Our data show that JAK3 mutations are drivers of T-ALL and require the cytokine receptor complex for transformation. These results warrant further investigation of JAK1/JAK3 inhibitors for the treatment of T-ALL.

INTRODUCTION

The JAK kinases (JAK1, JAK2, JAK3, TYK2) are a family of cytosolic tyrosine kinases that are essential for the signaling of cytokine receptors. All four JAK kinases share a common structure with receptor binding domains at the N-terminus and a regulatory pseudokinase domain and catalytic active kinase domains at the C-terminus. The two JAK kinase family members JAK1 and JAK3 are essential components of the heterodimeric interleukin-7 (IL7) receptor, in which the IL7Ralpha chain is bound by JAK1 and the common gamma chain (IL2RG) is bound by JAK3. Activation of the receptor by its ligand results in phosphorylation of both JAK1 and JAK3, with subsequent phosphorylation of STAT5 that translocates to the nucleus to regulate gene expression.[1] It is now well established that the correct activation of this IL7 receptor signaling pathway via JAK1 and JAK3 is critically important for the development of both B- and T-cells. Loss-of-function mutations in IL2RG, IL7R or JAK3 leads to severe immune deficiency.[2] Conversely, activating mutations in JAK1, JAK3 or IL7R have been reported in both B- and T-cell acute lymphoblastic leukemia (ALL) with mutations in any one of these genes found in up to 25% of T-ALL cases.[3-5]

Of the JAK family kinase mutations identified in hematological malignancies, JAK1 mutations are rare in childhood ALL, but are more frequent in adult ALL cases.[6,7] JAK3 mutations on the other hand have been identified in acute megakaryoblastic leukemia (AMKL), T-cell prolymphocytic leukemia and more recently in juvenile myelomonocytic leukemia (JMML) and natural killer T-cell lymphoma (NK/T-lymphoma).[8-13] The transformative potential of these JAK3 mutations has been confirmed in cell based assays *in vitro*, and the JAK3 A572V mutation identified in AMKL was also shown to confer features of megakaryoblastic leukemia and transform murine lymphoid cells *in vivo*.[10,14] However, systematic studies using both *in vitro* and *in vivo* models of newly identified JAK3 mutations are lacking, as too is knowledge on their potential mechanism of transformation.

In this work, twelve JAK3 pseudo-kinase and kinase domain mutations identified in T-ALL from massive parallel sequencing projects were studied using *in vitro* and *in vivo* experiments to assess their transformation potential. We report that the majority of these JAK3 mutants transform cells to cytokine independent growth *in vitro,* and that JAK1 kinase activity is required downstream of most JAK3 mutants. Moreover, we show that expression of JAK3 mutants in hematopoietic stem/progenitor cells leads to the development of a T-ALL like disease in a mouse bone marrow transplant model. We finally demonstrate that the use of tofacitinib, a JAK3 selective inhibitor, can reduce the leukemic burden within our *in vivo* bone marrow transplant model.

MATERIALS & METHODS

Expression plasmids

JAK3 wild type and mutant sequences were synthesized by GenScript. Kinase deficient JAK1 was provided by Dr. Haan.[15] The IL2RG expression plasmid was provided by Dr. Barata. JAK1 and IL7R constructs were described before.[16] All constructs were cloned into the MSCV-GFP or MSCV-puro vectors.

Cell culture, virus production, and retroviral transduction

293T cells were cultured in DMEM medium with 10% fetal calf serum (FCS). Virus production and retroviral transduction were performed as previously described.[17] Ba/F3 and MOHITO[18] cells were cultured in RPMI-1640 with 10-20% FCS and IL3 (10 ng/□L, Peprotech), IL-2 (25 ng/□L, Peprotech), and IL7 (50 ng/□L, Peprotech), respectively. For electroporation, $1.5x10^6$ Ba/F3 cells were resuspended in 400 □L of serum-free medium containing 200 nmol siRNA duplexes and then transferred to 4-mm cuvettes (Biorad). The electroporated cells were transferred to 12-well plates containing 2 mL of pre-warmed RPMI supplemented with 10% FCS. Growth reduction was analysed using the guava easyCyte Flow Cytometer (Millipore) 1h and 48h after electroporation. Human T-ALL cells were cultured as described.[19] Ethical approval and informed consent was obtained for the use of human T-ALL cells.

Western blotting

Cells were lysed in cold lysis buffer containing 5 mM NA_3VO_4 and protease inhibitors (Complete, Roche). The proteins were separated on NuPAGE NOVEX Bis-Tris 4%–12% gels (Invitrogen). Western blot analysis was performed using antibodies against JAK1, anti-phospho-tyrosine(4G10) (Millipore); AKT, phospho-AKT, phospho-ERK, JAK3, phospho-JAK3, STAT1, phospho-STAT1, STAT3, phospho-STAT3 phospho-STAT5, SRC, phospho-SRC (Cell Signaling); ERK, phospho-JAK1, STAT5 (Santa Cruz); and beta-actin (Sigma); secondary antibodies conjugated with horseradish peroxidase (GEHealthcare). Anti-phospho-JAK1 antibody was used to detect phosphorylated JAK1 and JAK3. Bands were visualized using a cooled charge-coupled device camera (ImageQuant LAS-4000, GEHealth Care).

Murine bone marrow transplantation

BALB/c mice were purchased from Charles River Laboratories. Male BALB/c mice were sacrificed and bone marrow cells were harvested from femur and tibia. Lineage negative cells were enriched (STEMCELL Technologies) and cultured in RPMI with 20% FCS, IL3 (10ng/mL, Peprotech), IL6 (10ng/mL, Peprotech), SCF (50ng/mL, Peprotech), and penicillin-

streptomycin. $1x10^6$ cells were transduced by spinoculation (90 min at 2500rpm; 8 µg/mL polybrene). Cells were washed in PBS and injected (1×10^6 cells/0.3 mL) into the lateral tail vein of sub-lethally irradiated (5 Gy) female BALB/c mice. Mice were housed in IVC cages and monitored daily. Approval of the local ethics committee was obtained.

Inhibitor experiments

Ba/F3 or MOHITO cells were seeded in 96-well plates ($1x10^5$ cells/ml) and treated with tofacitinib, ruxolitinib, or vehicle (DMSO). A quantitative evaluation of proliferation was done after 24h using ATPlite (PerkinElmer) and measured on the VICTOR X4 Reader (PerkinElmer). For *in vivo* experiments BALB/c mice were treated through oral gavage with tofacitinib (20-40mg/kg/day) or vehicle.

Flow cytometry analyses

Single-cell suspensions were prepared from peripheral blood, bone marrow, spleen, thymus and lymph nodes. Cells were analysed on a FACSCanto flow cytometer (BD Bio-sciences). Data were analysed with the FlowJo software (Tree Star).

RESULTS

1. JAK3 mutants identified in T-ALL transform Ba/F3 and MOHITO cells to cytokine independent growth

We recently reported the results from exome, transcriptome and targeted re-sequencing of 89 patients with T-ALL.[4,20,21] In this cohort of patients, we identified 12 mutations affecting the JAK3 protein in 10 of 89 diagnostic samples. To determine if JAK3 mutations identified in T-ALL are oncogenic driver mutations, we selected twelve mutants to study *in vitro* using the cytokine dependent Ba/F3 and MOHITO cell lines. These mutations included FERM/SH2 domain mutations (R272H, R403H), pseudokinase domain mutations (M511I, A572T, A573V, R657W, R657Q, V674A, V678M) and kinase domain mutations (L857Q, R925S, E1106G) (Figure 1A, Table S1).

The Ba/F3 and MOHITO cells were transduced by retroviral vectors encoding either JAK3 wild type or JAK3 mutants that co-express GFP. In all cases transduction efficiency exceeded 80% as measured by GFP expression. Of the JAK3 mutations screened, eight out of twelve were able to transform the Ba/F3 cells to IL-3 independent growth (Figure 1B). Of these eight JAK3 mutants, two mutants (V678M and A572T) were weaker in their ability to transform Ba/F3 cells to IL-3 independent growth. The remaining four JAK3 mutants,

including two kinase domain mutations (R925S and E1106G), and two FERM/SH2 domain mutants (R272H, R403H) did not transform Ba/F3 cells. Similarly, JAK3 wild type and the empty vector transduced cells were not able to induce autonomous cell growth. Similar results were observed in the MOHITO cell line, as the same JAK3 mutants were able to stimulate the proliferation and survival of the cells in the absence of IL-2 and IL-7 cytokines (Figure 1C). Transformation was further demonstrated by the fact that the transformed MOHITO cells outcompeted the non-transduced cells, as only GFP positive cells were able to grow in the absence of cytokines (data not shown).

2. JAK3 mutants activate JAK1, STAT5 and ERK

We next sought to identify the downstream signaling pathways activated by JAK3 mutants in the transformed Ba/F3 cell lines. We first analyzed the levels of JAK3 phosphorylation by Western blot using a phospho-JAK antibody raised against JAK1 Tyr1022/1023 but recognizes both pJAK1 and pJAK3. This showed JAK3 phosphorylation was variable between the different JAK3 mutants with some mutants (i.e. R657Q, L857Q) showing a clear phosphorylation of JAK3 whilst other mutants showed only weak JAK3 phosphorylation (Figure 2A). To determine whether other tyrosine residues were potentially phosphorylated, a JAK3 immunoprecipitation followed by immunoblot detection with a general anti-phosphotyrosine antibody was carried out. This confirmed that all JAK3 mutants had phosphorylated tyrosine residues present, with strong tyrosine phosphorylation evident for A573V, R657Q, moderate for V674A, L857Q, V678M, and weak for A572T and M511I mutants (Figure 2B).

We also observed that JAK1 phosphorylation was present in Ba/F3 cells expressing the different JAK3 mutants. Although the level of JAK1 phosphorylation was lower in the JAK3 mutant transformed cells compared to cells transformed by the constitutively active JAK1 A634D mutant (Figure 2A). In addition, phosphorylation of STAT5 and ERK was detected in all Ba/F3 cells expressing JAK3 mutants (Figure 2C). Notably, we did not observe phosphorylation of STAT1, STAT3 or AKT in any of the transformed Ba/F3 cells expressing JAK3 mutants (Figure S1). These results were also confirmed in the MOHITO cell line (data not shown). Taken together, these results show that cytokine independent growth of Ba/F3 or MOHITO expressing JAK3 mutants was concomitant with increased phosphorylation of JAK1, STAT5 and ERK.

3. JAK1 is essential for the transforming properties of JAK3 pseudokinase domain mutants

We then investigated whether the transforming capacity of JAK3 mutants was dependent on the presence of the cytokine receptor complex (e.g. IL7R complex). To test this, we used 293T cells that lack endogenous cytokine receptor expression. Cells transfected with JAK3 mutants alone did not result in JAK3 phosphorylation (Figure 3A). Co-expression of JAK3 mutants with IL2RG was sufficient to obtain JAK3 phosphorylation (Figure 3B, 3C), while activation of STAT5 required the co-expression of JAK3 mutants with IL2RG and JAK1 (Figure 3B) or IL2RG and IL7R (Figure 3C). The reconstitution of the entire receptor complex yielded the strongest activation of both JAK3 and STAT5. These data indicate that JAK3 mutants cannot function in the absence of additional receptor components. For wild type JAK3, co-expression of the complete receptor complex (IL7R, IL2RG, JAK1, JAK3) with IL7 ligand stimulation was required to obtain STAT5 phosphorylation (Figure 3C). Those JAK3 mutants unable to transform Ba/F3 cells, did not cause JAK1 or JAK3 phosphorylation in 293T cells and were predicted as 'neutral' variants using PROVEAN[22] analysis (Figure S2), adding support that the R925S and E1106G are non-transforming passenger mutations.

To confirm the essential role of JAK1 for the signaling of the JAK3 mutants, we downregulated the expression of endogenous murine Jak1 or Jak2 in the Ba/F3 cells. Knockdown of Jak expression was confirmed by qPCR and consistently showed knockdown efficiency of >90% (Figure 3D). For the pseudokinase domain mutants, siRNA mediated knockdown of Jak1 expression resulted in a >90% decrease in cell proliferation. This was not the case for cells transformed by the kinase domain JAK3 L857Q mutant where loss of JAK1 did not significantly alter cell proliferation. For both pseudokinase and kinase domain mutants, loss of endogenous Jak2 expression did not affect cell proliferation (Figure 3D).

To determine if the observed dependence on Jak1 was due to the complete loss of Jak1 protein or only Jak1 kinase activity, we performed rescue experiments with either wild type human JAK1 or a kinase-dead JAK1 mutant. Expression of the siRNA resistant human JAK1 but not the kinase dead JAK1 could rescue the block in proliferation upon Jak1 knockdown (Figure 3D). Taken together, these results indicate that JAK1 kinase activity is essential for the transforming capacity of JAK3 pseudokinase domain mutants, but not for the kinase domain mutant L857Q.

4. JAK3 mutant transformed cells are sensitive to JAK kinase inhibitors

To determine the sensitivity of the different JAK3 mutants to JAK kinase inhibitors, and to further confirm the requirement of JAK1, we treated the transformed Ba/F3 and MOHITO

cells with tofacitinib, a JAK3 selective inhibitor[23], or ruxolitinib, a JAK2/JAK1 selective inhibitor.[24] Cells were first treated with increasing concentrations of the JAK3 selective inhibitor tofacitinib or vehicle (DMSO) with a quantitative evaluation of proliferation performed at 24h post-treatment. All Ba/F3 cells transformed by JAK3 mutants were sensitive to tofacitinib treatment, with IC50 values ranging between 246 nM and 408 nM (Figure 4A). Wild type Ba/F3 cells which are dependent on IL3/JAK2 signaling had a significantly higher IC50 of 1400 nM with these high concentrations speculated to also have an inhibitory effect on JAK2 kinase activity. The proliferation of cells transformed by FLT3-ITD kinase, which are independent of JAK activity, was not affected by tofacitinib treatment. Similar results were obtained for transformed MOHITO cells (Figure S3-S4).

Confirmation that JAK1 is required for the transforming capacities of JAK3 mutant proteins was also evident after cells were treated with the JAK1 selective inhibitor ruxolitinib. Ba/F3 cells dependent on the expression of JAK3 pseudokinase domain mutants were sensitive for low doses of ruxolitnib treatment, with IC50 values ranging from 120 nM to 398 nM. This is in agreement with our earlier results, where all pseudokinase domain mutants require JAK1 kinase activity for their transforming capacities. The cells expressing the JAK3 L857Q kinase domain mutant were less sensitive with an IC50 value of 855 nM confirming JAK3 L857Q is less dependent on JAK1 kinase activity. The inhibitory effects observed at higher ruxolitinib concentration in the L857Q cells is speculated to be due to inhibition of the JAK3 kinase by ruxolitinib.[24] Similar results were obtained for transformed MOHITO cells (Figure S5-S6). Finally, we also treated the Ba/F3 cells with low doses of tofacitinib, ruxolitinib or a combination of both drugs. This resulted in the synergistic inhibition of cells dependent on JAK1 cells but not in cells independent of JAK1 activity (e.g. L857Q mutant) (Figure S7), confirming again that pseudokinase domain mutants are dependent on JAK1 activity.

To determine whether treatment of JAK3 mutant transformed Ba/F3 cells also decreases downstream signaling, cells were treated with increasing concentration of tofacitinib (300, 600, 1200nM), ruxolitinib (125, 500, 2000nM) or vehicle (DMSO) for 90 minutes before cell lysis. In all cases STAT5 phosphorylation significantly decreased with increasing tofacitinib concentration (Figure 4C). Once again, changes in STAT5 phosphorylation for the kinase domain mutant L857Q were less marked after ruxolitinib treatment and confirm that this mutant can signal to STAT5 independent of Jak1 (Figure 4D). We used here STAT5 phosphorylation as read-out, as effects on JAK1/JAK3 phosphorylation by JAK kinase inhibitors are generally modest, as also previously described.[25] In primary human T-ALL cells with the JAK3 M511I mutation we observed inhibition of STAT5 phosphorylation upon treatment with tofacitinib or ruxolitinib, but no effect was seen on phosphorylation of ERK

(Figure 5A). This may be due to the presence of other mutations in the T-ALL sample, which leads to the activation of the ERK pathway in a JAK independent manner. These data are also in line with our observation that the proliferation of the JAK3 M511I mutant T-ALL cells was inhibited by tofacitinib or ruxolitinib, while the proliferation of human T-ALL cells that did not express mutant JAK3 were not affected by the presence of JAK inhibitors (Figure 5B).

5. JAK3 mutants cause T-cell acute lymphoblastic leukemia in a bone marrow transplant mouse model

Having established that the JAK3 mutants could transform cells *in vitro*, we used a mouse bone marrow transplant model to determine whether they were also transforming *in vivo.* Our initial assessment focused on the JAK3 M511I pseudokinase domain mutation because it was the most common JAK3 mutation identified in T-ALL. In this model, hematopoietic progenitor cells transduced with retroviral constructs expressing JAK3 M511I and GFP were injected in the tail vein of irradiated female recipient BALB/c animals. Animals initially developed a lymphoproliferative disease over the first 12 weeks characterized by moderate increased white blood cell (WBC) counts (range: 10,000 and 20,000/microliter) (Figure 6A). After this initial lymphoproliferative stage, all animals expressing JAK3 M511I progressed to an acute phase. The acute phase was fatal and was characterized by a rapid rise in WBC counts (range: 50,000 to 300,000/microliter) between 14 to 28 weeks post-transplant. Mice transplanted with wild type JAK3 expressing cells did not develop any disease (Figure 6A,B).

All mice showed accumulation of CD8 single positive cells in the peripheral blood with the majority of mice succumbing to the disease between 100-200 days post-transplant (Figure 6B). At end stage disease there was a significant increase in spleen and thymus weight (Figure 6C). Spleen, thymus, lymph nodes and bone marrow were infiltrated by TdT positive immature T-cells (Figure 6D). GFP positive blast cells in all organs also had variable expression of TCRβ and CD3. Specific phenotypic analysis of the bone marrow showed that 5 out of 16 mice had more than 50% blasts that were CD8 single positive whilst 11 out of 16 mice the bone marrow contained a CD4/CD8 double negative population (Figure 6E), corresponding to an accumulation of more immature T-cells. Mouse leukemia cells showed phosphorylation of JAK1/JAK3, STAT5 and ERK (Figure S8).

Notably, the leukemic cells were also transplantable, and caused a rapid development of T-ALL and earlier onset of disease in the secondary and tertiary transplanted animals (Figure 6B). In four out of seven JAK3 M511I secondary transplant experiments, recipient mice developed an acute leukemia within 100 days post transplant. All secondary transplanted mice had an accumulation of TCRβ/CD3 negative cells, indicating that the acute phase seen

in primary transplant experiments was associated with loss of differentiation and accumulation of more immature T-cells (Figure S9-S12). Leukemic cells with high TCRβ expression failed to induce leukemia in recipient mice, indicating that more differentiated cells are not able to induce leukemia in a secondary transplant. Analysis of TCRβ rearrangement showed oligoclonal origin of the primary leukemias, while TCRβ was not rearranged in secondary leukemias, in agreement with the immunophenotyping (Figure S11-S13). Three heterozygous mutations (*Notch1* L1668P, Notch1 indel(R2361), *Pten G165E*) were identified in primary leukemias (Figure S13). Together, these data support a clonal nature of the mouse leukemias.

In addition to JAK3 M511I, other JAK3 mutations (A573V, L857Q, V674A and R657Q) were tested in the BALB/c bone marrow transplant model. Mice transplanted with cells expressing JAK3 A573V or V674A showed a gradual increase of the WBC count and developed a similar T-ALL like disease as observed for the JAK3 M511I mutant. In contrast to JAK3 A573V and V674A, mice transplanted with cells expressing JAK3 L857Q or R657Q showed no increase in WBC count compared to the control mice, but did present with severe splenomegaly and lymphadenopathy. Expression of JAK3 L857Q caused severe thymus hyperplasia, while the JAK3 R657Q mutant caused B-cell leukemia. Detailed results are presented in supplement (Figure S14).

6. JAK3 M511I mutant cells respond to tofacitinib treatment *in vivo*

Our *in vitro* data showed that tofacitinib, a selective JAK3 inhibitor, potently inhibited all tested JAK3 mutants in cell based assays. Tofacitinib does not show potent activity against JAK2, and was recently approved by the FDA for the treatment of rheumatoid arthritis. We therefore selected tofacitinib to determine the *in vivo* response of JAK3 driven leukemia to JAK inhibitor treatment. We used the primary JAK3 M511I transplant model and the secondary JAK3 M511I transplant model, and treated those animals by oral gavage.

In a first experiment, primary transplanted animals were treated with tofacitinib (30 mg/kg/day) for 6 consecutive days. Treatment started 82 days after transplantation, at the time the mice had increased WBC counts. All mice showed a decrease in WBC count during treatment, and again an increase in WBC count after treatment was stopped, demonstrating a clear effect of tofacitinib on the proliferation of the leukemia cells *in vivo* (Figure 7A). In a second experiment, primary transplanted mice were treated with tofacitinib (40 mg/kg/day; n=5) or placebo (n=6) for 5 weeks. Treatment started 48 weeks after bone marrow transplant, at which time WBC values had already increased. Mice receiving tofacitinib treatment showed a decrease in WBC count at the end of the experiment, while WBC counts

of placebo treated mice were all increased (Figure 7B).

In a third experiment, secondary transplanted mice were treated for 14 days with tofacitinib (20 mg/kg/day; n=5) or placebo (n=5). The WBC count of placebo treated mice increased significantly during the 14 days of treatment, while the overall increase in WBC count for tofacitinib treated mice was lower compared to the placebo treated mice (Figure 7C), indicating that tofacitinib has an inhibitory effect on the leukemia cells. In addition, mice treated with tofacitinib showed reduced spleen and thymus weight compared to mice treated with placebo (Figure 7D). Histopathological analysis of spleen and thymus documented clear induction of apoptosis in the tofacitinib treated animals, as observed by anti-cleaved caspase 3 staining and the typical 'starry sky' pattern in H&E stained tissue sections caused by the increased number of macrophages clearing the apoptotic cells (Figure 7E).

DISCUSSION

Protein tyrosine kinases are attractive targets for therapy in oncology, as these proteins are often mutated and activated in cancer, and the tumor cells become addicted to these activated signaling pathways for their proliferation and survival. Also in leukemia, tyrosine kinases such as ABL1, FLT3, PDGFR and JAK2 have been extensively studied as possible targets for therapy, with the successful application of kinase inhibitors for the treatment of CML as one of the most important breakthroughs in this area.[26]

The JAK3 tyrosine kinase was recently found to be recurrently mutated in JMML, NK cell lymphoma and T-ALL, with mutations mainly clustering in the pseudokinase and kinase domains. Earlier studies had also reported rare mutations of JAK3 in AML. Together, these studies identify JAK3 as a possible new target for therapy in various hematological malignancies. To further study the role of JAK3 as a possible therapeutic target, we performed a detailed analysis of the *in vitro* and *in vivo* transforming properties of a selection of 12 JAK3 mutations identified in T-ALL.

Using *in vitro* and *in vivo* models, we have shown that most JAK3 mutants identified in T-ALL patient samples were capable of conferring cytokine independent growth to cell lines *in vitro* and to cause leukemia *in vivo*. Few of the mutants were lacking transforming potential in these experiments, illustrating that results from sequencing always need to be confirmed by functional assays to distinguish driver mutations from passenger mutations.

Based on the Ba/F3 and MOHITO data we can also distinguish strong JAK3 mutants from weaker transforming mutants such as the A572T and V678M mutants. It is of interest to note that these two weaker alleles were identified together with a strong JAK3 mutation M511I or L857Q, respectively (supplementary table 1). The fact that some T-ALL cases harbor two transforming JAK3 mutations suggests that T-ALL leukemia cells that are dependent on a JAK3 mutation can obtain an additional proliferation advantage by mutating the second JAK3 allele, similar as to what has been observed for the JAK2 V617F mutation in myeloproliferative neoplasms.[27]

Several lines of evidence suggest that JAK1 is an essential kinase required downstream of JAK3 mutants. Indeed, a close relationship exists between JAK1 and JAK3 at the normal IL7 receptor (and other type I cytokine receptors), where both kinases phosphorylate and activated each other upon stimulation of the receptor. We observed that in the Ba/F3 cells transformed by JAK3 mutants, JAK1 was always phosphorylated, and that transformation by JAK3 mutants was lost upon knock-down of Jak1. Remarkably, the dependency on JAK1 was observed for all pseudokinase domain JAK3 mutants, but not for the JAK3 kinase domain mutant (L857Q). These observations are also in line with the data obtained with JAK kinase inhibitors, which show that the JAK3 pseudokinase mutants are more sensitive to ruxolitinib, a kinase inhibitor targeting JAK2 and JAK1, than the JAK3 kinase domain mutant (L857Q).[24] In contrast, all JAK3 mutants showed equal sensitivity to tofacitinib, a JAK3 selective inhibitor.[23] These findings indicate that different JAK3 mutations may have different signaling properties, which may affect their sensitivity to JAK kinase inhibitors.

Several JAK kinase inhibitors are currently under development for the treatment of myeloproliferative neoplasms with JAK2 mutation or for the treatment of auto-immune diseases. Our data show that both JAK1 selective and JAK3 selective inhibitors can have inhibitory effects on JAK3 mutation positive leukemias, but that different JAK3 mutants show different signaling properties that affects their sensitivity to the various JAK inhibitors. Further pre-clinical and clinical studies are warranted and may bring new treatment opportunities for hematological malignancies with JAK3 mutation.[11,13,20,28,29]

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Authorship contribution

SD, LC, CEDB, OG, NM, KJ, EG, VG performed experiments and analyzed data; TT supervised histopathology and analyzed data; SDem, GH, MF, SA contributed to sequence analysis; JPM contributed valuable reagents; SD, CEDB, JC wrote the manuscript; JC supervised the study.

Conflict of interest disclosure

The authors declare no competing financial interests.

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Figure legends

Figure 1. JAK3 mutants transform Ba/F3 and MOHITO cells to cytokine independent growth. (**A**) Schematic representation of JAK3 protein and its main domains; the Four-point-one, Ezrin, Radixin, Moesin (FERM) domain, the Src homology-2 (SH2) domain, the pseudokinase and kinase domain. Mutations which are studied in this work are shown. (**B,C**) Proliferation curve of Ba/F3 (**B**) or MOHITO (**C**) cells expressing various JAK3 mutants, JAK3 wild type or empty vector, in the absence of cytokines. Mutations that did not stimulate proliferation more than wild type JAK3 were considered as not transforming mutants and are indicated with asterisk (*).

Figure 2. JAK3 mutants signal through JAK1, STAT5 and ERK in a cytokine independent way. (**A**) Western blot analysis of whole cell lysates of Ba/F3 cells transformed by JAK3 mutants. Phosphorylation of JAK1 was detected for all JAK3 mutants. JAK3 phosphorylation was clearly detected for some, but not all JAK3 mutant proteins, most likely due to the specificity of the used antibody. JAK3 protein expression was detected with a human specific antibody, not recognizing the endogenous JAK3 expression. (**B**) JAK3 phosphorylation could be detected for all JAK3 mutants after immunoprecipitation and detection with a phospho-tyrosine antibody (4G10). (**C**) All transforming JAK3 mutants were able to phosphorylate downstream signaling components STAT5 and ERK.

Figure 3. JAK1 kinase activity is essential for the transforming properties of JAK3 mutants. (**A**) Western blot detection of JAK3 mutants expressed in 293T cells. (**B,C**) Western blot analysis of whole cell lysates after reconstitution of the IL7 receptor signaling complex in 293T cells. The 293T cells were transiently transfected with the constructs as indicated. (**D**) Graph shows relative proliferation of Ba/F3 cells expressing JAK3 M511I, R657Q or L857Q 48 hours after knockdown of endogenous Jak1 or Jak2 compared to scrambled siRNA. (**E,F**) Rescue of the Jak1 knockdown, through the expression of human JAK1 (**E**) or kinase dead JAK1 (**F**). Relative proliferation is shown 48 hours after knockdown of endogenous Jak1. Knock down efficiency was determined by qPCR for all cell lines, results are shown for one cell line, but is representative for all cell lines.

Figure 4. Cells dependent on JAK3 mutants are sensitive to JAK3 and JAK1 inhibition. (**A,B**) Relative proliferation of Ba/F3 cells transformed by JAK3 mutants or FLT3 ITD or wild type Ba/F3 cells stimulated with IL3 after treatment with tofacitinib or ruxolitinib, respectively. Proliferation was compared to proliferation of cells after vehicle (DMSO) treatment. Full dose response curves for all JAK3 mutants are shown in supplement. (**C,D**) Western blot analysis of Ba/F3 cells expressing JAK3 mutants after 90 minutes treatment with tofacitinib or ruxolitinib.

Figure 5. *Ex vivo* treatment of primary human T-ALL cells with JAK3 M511I mutation. (**A**) Western blot analysis of xenograft derived cells which express JAK3 M511I were cultured *ex vivo* and treated for 90 minutes with tofacitinib, ruxolitinib or vehicle (DMSO). (**B**) Graph shows relative proliferation after 48 hours treatment with JAK inhibitors of human T-ALL xenograft derived cells.

Figure 6. Expression of JAK3 M511I in bone marrow cells of Balb/c mice cause a T-lymphoproliferative disease that progress to T-ALL. (**A**) Evolution of the white blood cell (WBC) count in JAK3 M511I animals. WBC was measured every two weeks. The upper limit of normal WBC count (10,000/mm^3) is indicated by a dashed line. (**B**) Kaplan Meier survival curve of BALB/c mice receiving a bone marrow transplantion of lineage negative cells expressing JAK3 M511I. Mice transplanted with cells expressing wild type JAK3 did not develop any hematological abnormalities nor signs of disease (**C**) Spleen and thymus weight of mice transplanted with cells expressing JAK3 M511I compared to mice transplanted with

JAK3 wild type cells. Significance was determined by t-test. (**D**) H&E staining of spleen, bone marrow, thymus and lymph node. TdT staining of thymus and lymph node. Scale bar indicates 100 micrometer. (**E**) Analysis of the bone marrow cells of diseased animals by flow cytometry with anti-CD4 and anti-CD8 antibodies. Pathology images were obtained using a Leica DM2500 microscope and a Leica DFC290HD camera and analyzed using the Leica Application suite LAS v4.1 software. Images were processed with Adobe Photoshop CS5.

Figure 7. JAK3 M511I cells are sensitive to the JAK3 inhibitor tofacitinib in vivo. (A) 4 BALB/c mice which received a primary transplant of cells expressing JAK3 M511I, were treated for 6 days with tofacitinib, two times a day with a total concentration of 30mg/kg/day. WBC count was determined before, during and after treatment. Graph shows WBC count over time, indicating days post transplant. (**B**) 11 BALB/C mice received a primary transplant of cells expressing JAK3 M511I. Mice were randomly divided into two groups. One group was treated with tofacitinib for 5 weeks, twice daily, with a total concentration of 40mg/kg/day. The other group received vehicle (DMSO) treatment. WBC count was determined at the start and the end of treatment. Graph shows difference in WBC count over the 5 weeks of treatment. (**C**) 10 BALB/c mice received a secondary transplant of cells expressing JAK3 M511I. Afterwards, mice were randomly divided into two groups. Both groups were treated for a period of 14 days, one group with tofacitinib, the second group with vehicle (DMSO). Treatment was performed two times a day with a total concentration of 20mg/kg/day. WBC count was determined at the start and the end of the experiment. Graph shows the difference in WBC count over the 14 days of treatment. (**D**) Graphs shows spleen, thymus and lymph node weight of secondary transplanted mice with JAK3 M511I, after 18 days of treatment with tofacitinib or vehicle. Significance was determined by T-test. (**E**) Cleaved caspase 3 staining of spleen and thymus samples retrieved from the experiment shown in panel C. H&E staining of thymus after treatment with placebo or tofacitinib. Scale bars indicate 100 micrometer. Pathology images were obtained using a Leica DM2500 microscope and a Leica DFC290HD camera and analyzed using the Leica Application suite LAS v4.1 software. Images were processed with Adobe Photoshop CS5.

Figure 1

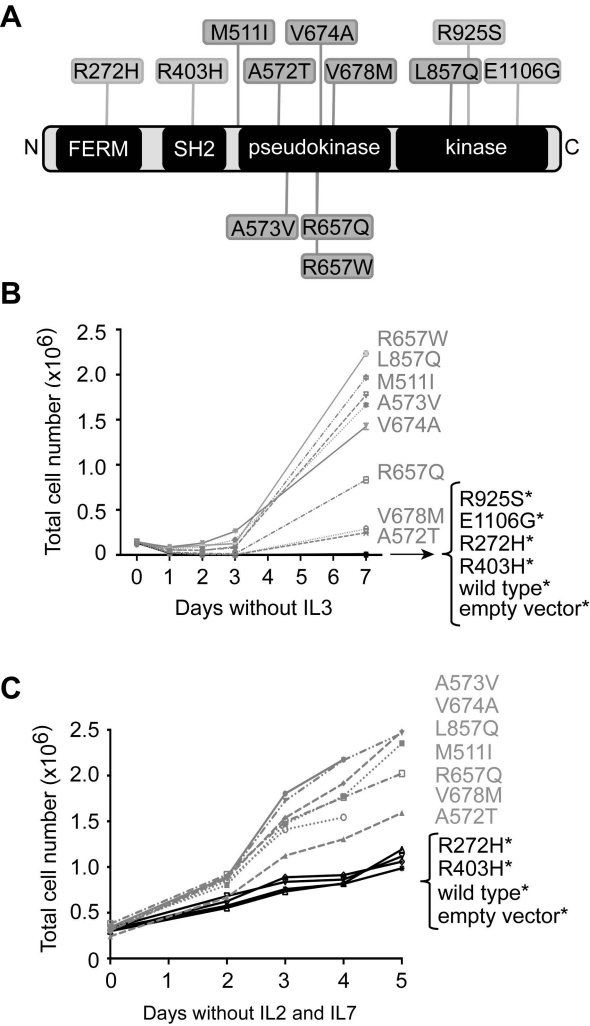

A
M511I
V674A
R925S
R272H
R403H
A572T
V678M
L857Q
E1106G
N
FERM
SH2
pseudokinase
kinase
C
A573V
R657Q
R657W
B
Total cell number (x10^6)
2.5
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5 6 7
Days without IL3
R657W
L857Q
M511I
A573V
V674A
R657Q
V678M
A572T
R925S*
E1106G*
R272H*
R403H*
wild type*
empty vector*
C
Total cell number (x10^6)
2.5
2.0
1.5
1.0
0.5
0
0 1 2 3 4 5
Days without IL2 and IL7
A573V
V674A
L857Q
M511I
R657Q
V678M
A572T
R272H*
R403H*
wild type*
empty vector*

Figure 2

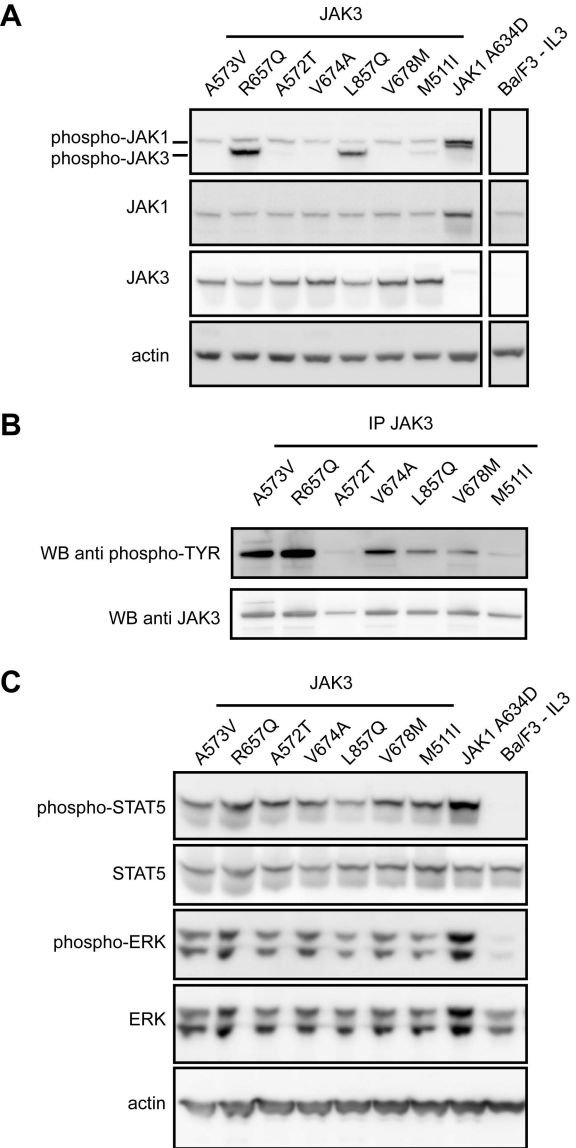

A
JAK3
A573V
R657Q
A572T
V674A
L857Q
V678M
M511I
JAK1 A634D
Ba/F3 - IL3
phospho-JAK1
phospho-JAK3
JAK1
JAK3
actin
B
IP JAK3
A573V
R657Q
A572T
V674A
L857Q
V678M
M511I
WB anti phospho-TYR
WB anti JAK3
C
JAK3
A573V
R657Q
A572T
V674A
L857Q
V678M
M511I
JAK1 A634D
Ba/F3 - IL3
phospho-STAT5
STAT5
phospho-ERK
ERK
actin

Figure 3

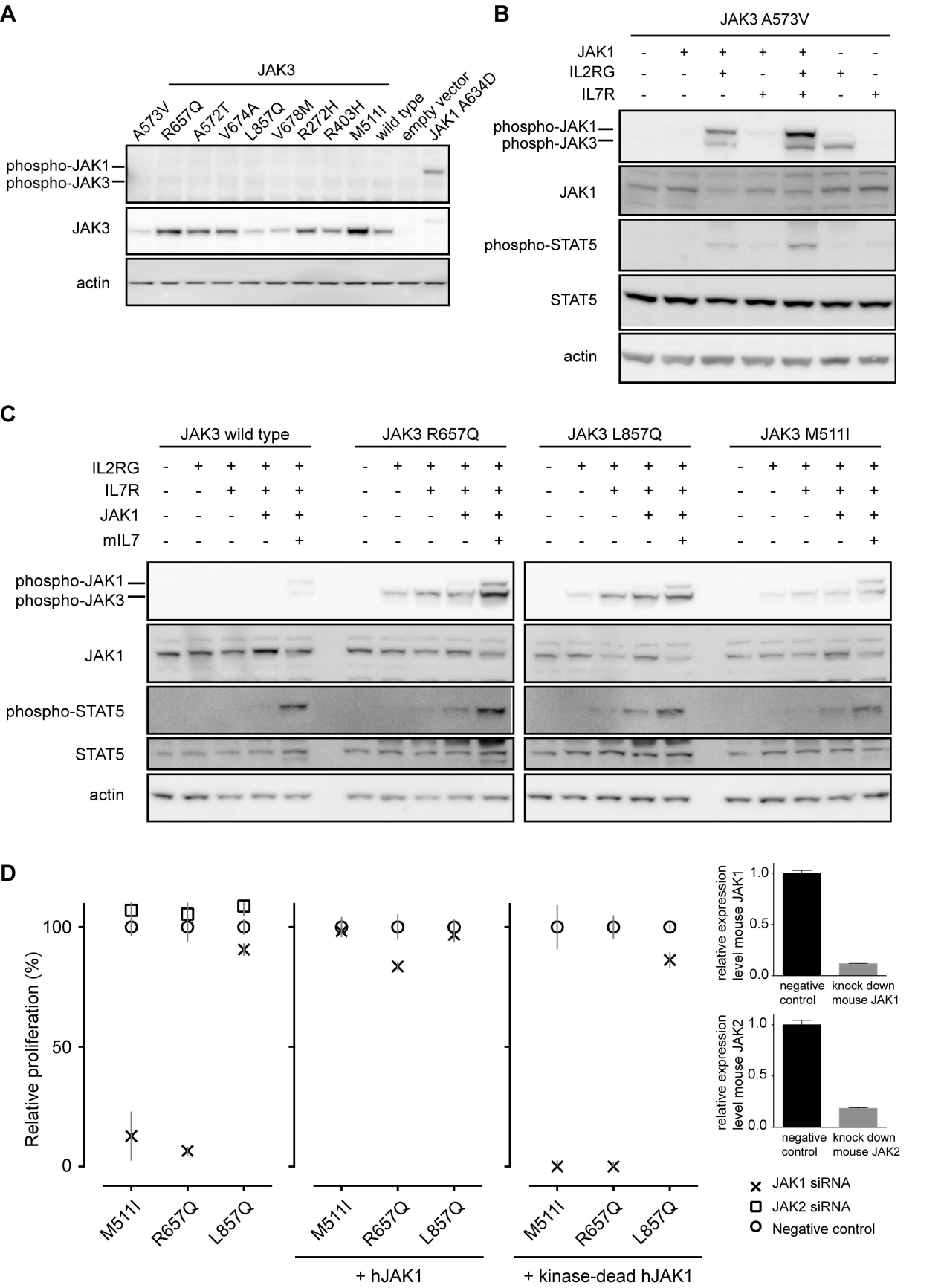
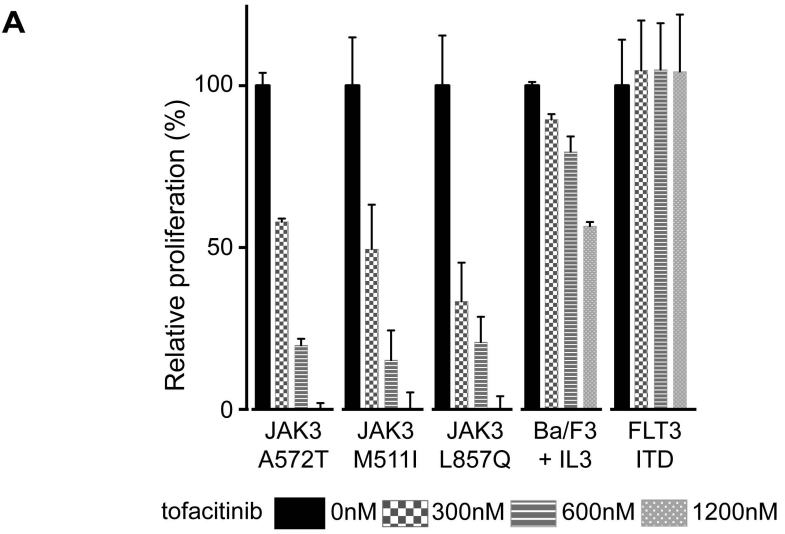
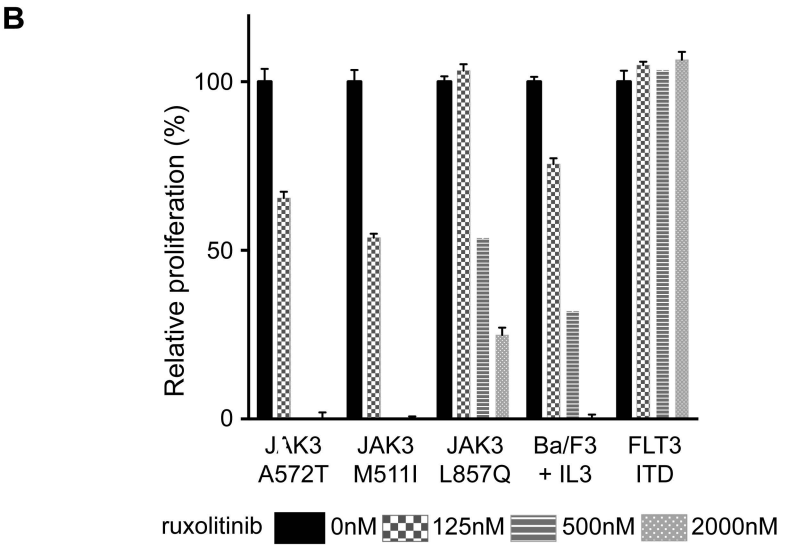
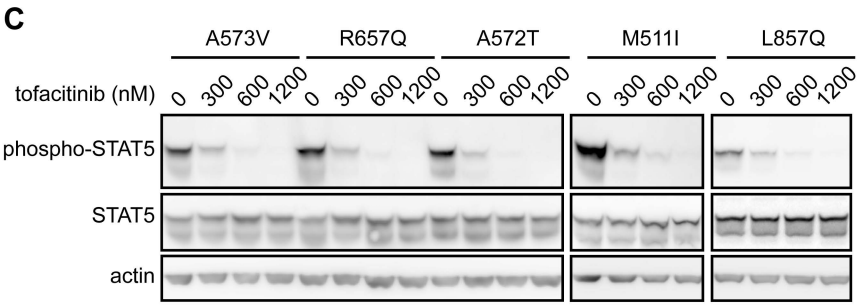

A
JAK3
A573V
R657Q
A572T
V674A
L857Q
V678M
R272H
R403H
M511I
wild type
empty vector
JAK1 A634D
phospho-JAK1
phospho-JAK3
JAK3
actin
B
JAK3 A573V
JAK1
IL2RG
IL7R
phospho-JAK1
phosph-JAK3
JAK1
phospho-STAT5
STAT5
actin
C
JAK3 wild type
JAK3 R657Q
JAK3 L857Q
JAK3 M511I
IL2RG
IL7R
JAK1
mIL7
phospho-JAK1
phospho-JAK3
JAK1
phospho-STAT5
STAT5
actin
D
Relative proliferation (%)
100
50
0
M511I
R657Q
L857Q
+ hJAK1
+ kinase-dead hJAK1
relative expression level mouse JAK1
1.0
0.5
0.0
negative control
knock down mouse JAK1
relative expression level mouse JAK2
1.0
0.5
0.0
negative control
knock down mouse JAK2
JAK1 siRNA
JAK2 siRNA
Negative control

Figure 4


A
Relative proliferation (%)
100
50
0
JAK3 A572T
JAK3 M511I
JAK3 L857Q
Ba/F3 + IL3
FLT3 ITD
tofacitinib
0nM
300nM
600nM
1200nM


B
Relative proliferation (%)
100
50
0
JAK3 A572T
JAK3 M511I
JAK3 L857Q
Ba/F3 + IL3
FLT3 ITD
ruxolitinib
0nM
125nM
500nM
2000nM


C
A573V
R657Q
A572T
M511I
L857Q
tofacitinib (nM)
0 300 600 1200
phospho-STAT5
STAT5
actin

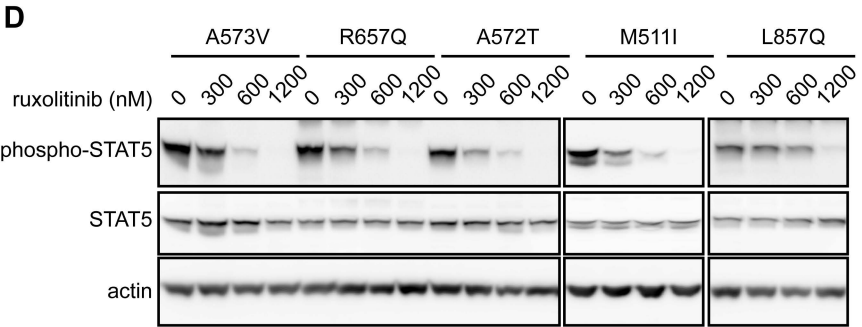
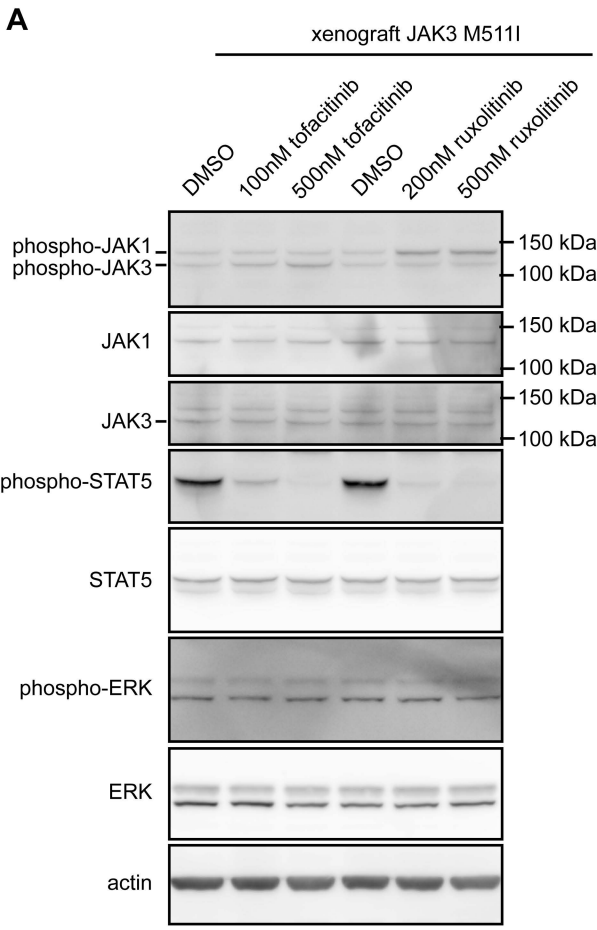

D
A573V
R657Q
A572T
M511I
L857Q
ruxolitinib (nM)
0 300 600 1200
phospho-STAT5
STAT5
actin

Figure 5


A
xenograft JAK3 M511I
DMSO
100nM tofacitinib
500nM tofacitinib
DMSO
200nM ruxolitinib
500nM ruxolitinib
phospho-JAK1
phospho-JAK3
JAK1
JAK3
150 kDa
100 kDa
phospho-STAT5
STAT5
phospho-ERK
ERK
actin

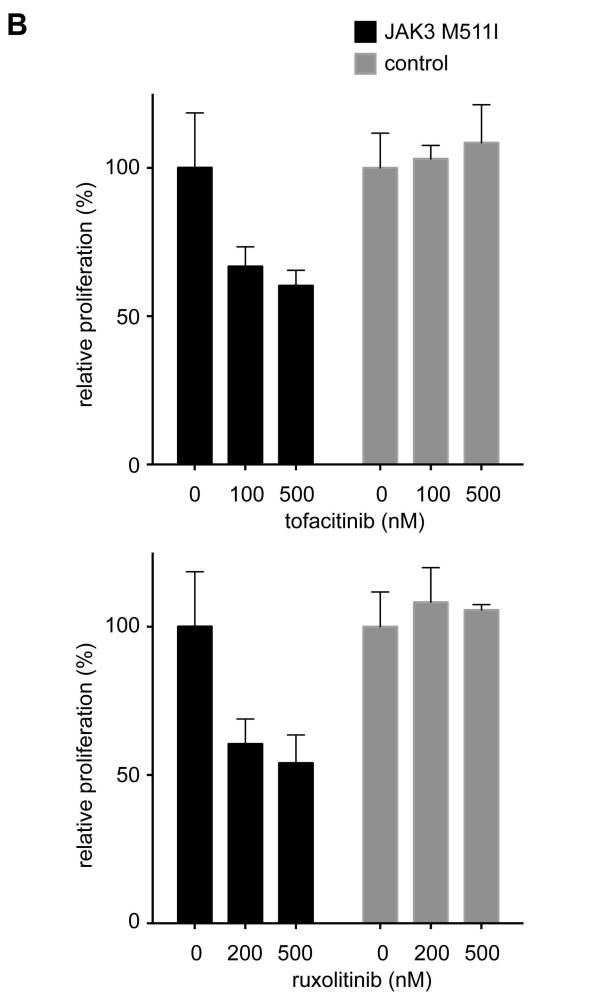

B
JAK3 M511I
control
relative proliferation (%)
100
50
0
0
100
500
0
100
500
tofacitinib (nM)
relative proliferation (%)
100
50
0
0
200
500
0
200
500
ruxolitinib (nM)

Figure 6

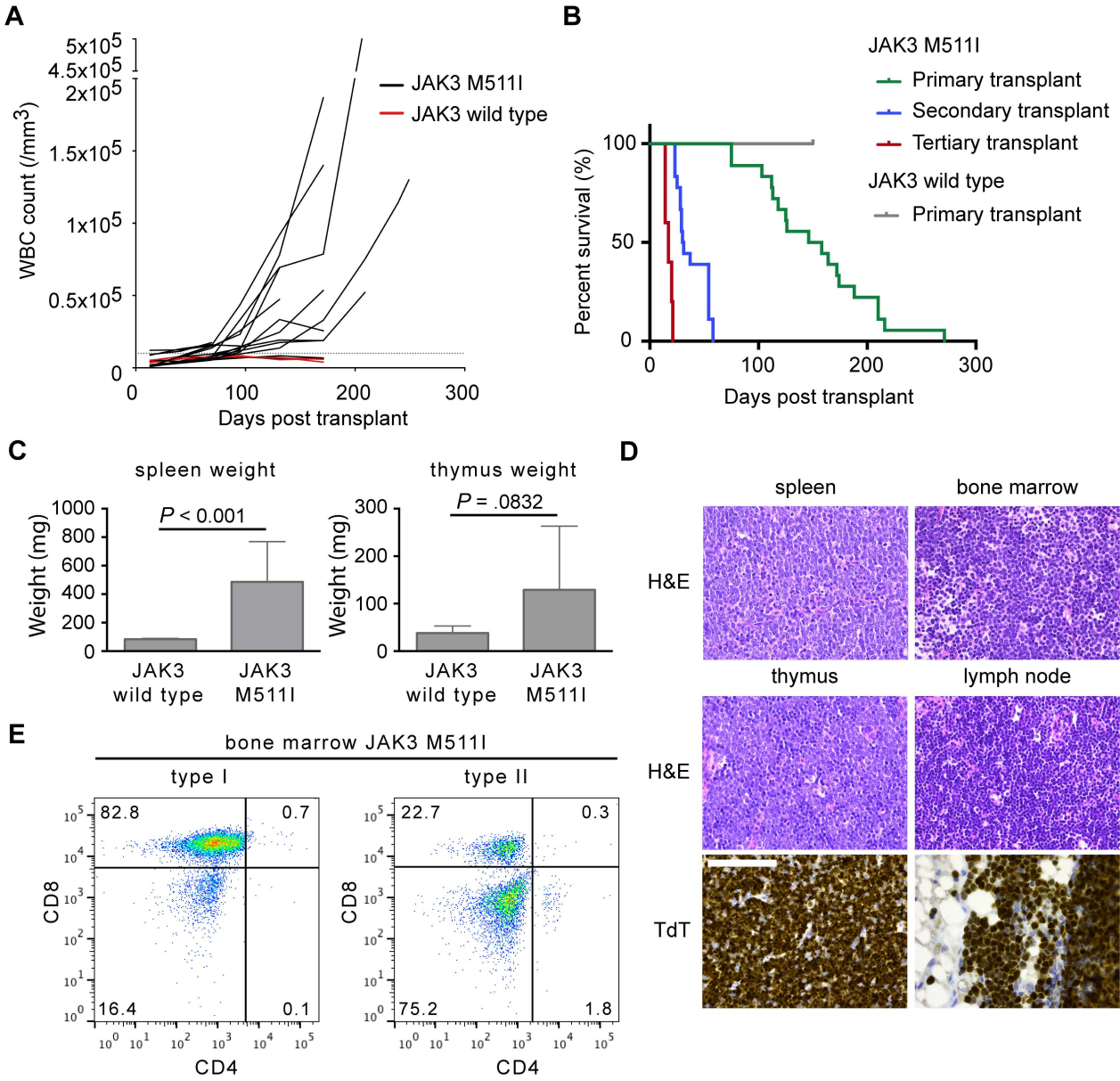
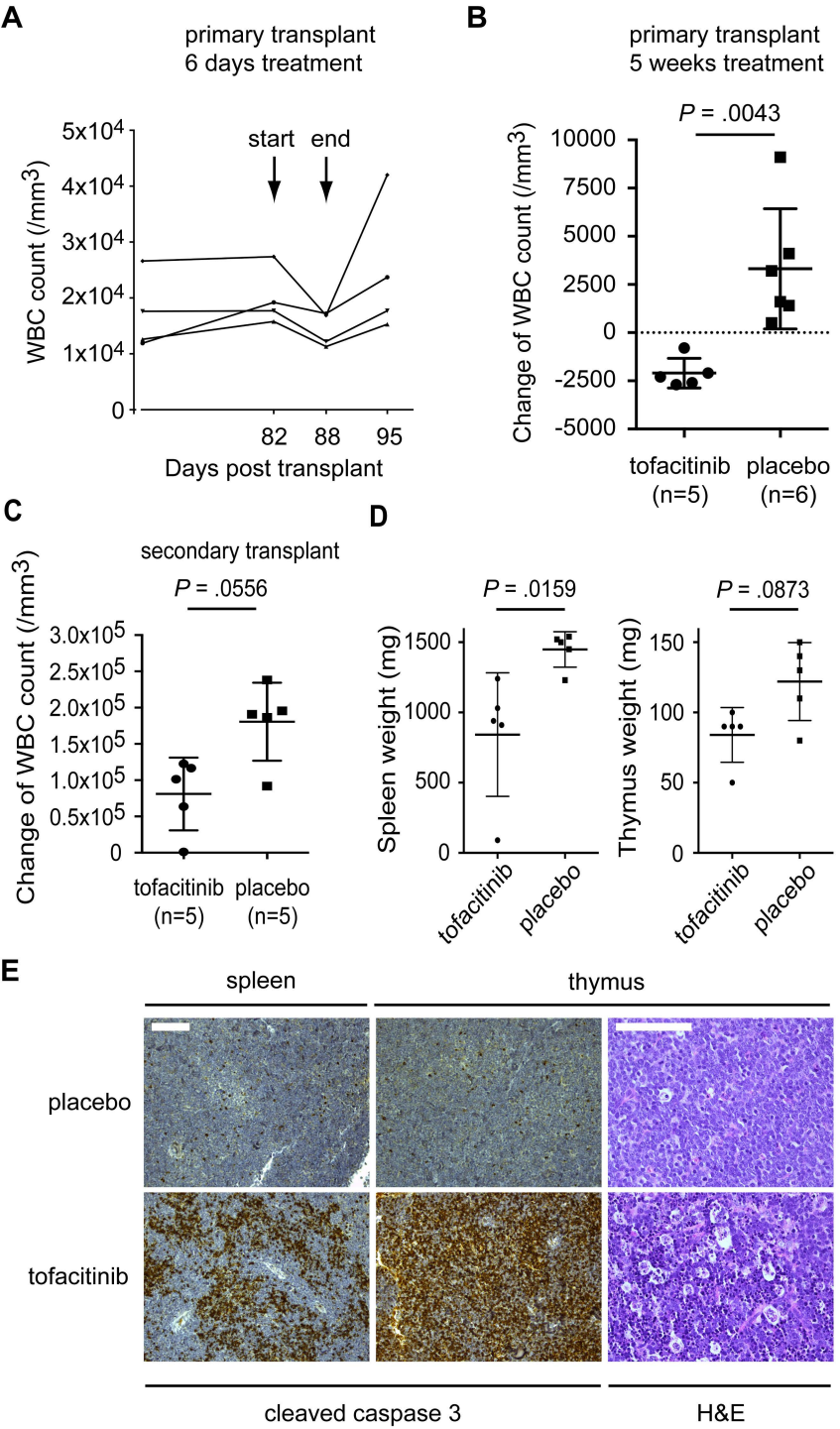

A
WBC count (/mm3)
5x10^5
4.5x10^5
2x10^5
1.5x10^5
1x10^5
0.5x10^5
0
0
100
200
300
Days post transplant
JAK3 M511I
JAK3 wild type
B
Percent survival (%)
100
50
0
0
100
200
300
Days post transplant
JAK3 M511I
Primary transplant
Secondary transplant
Tertiary transplant
JAK3 wild type
Primary transplant
C
spleen weight
P < 0.001
Weight (mg)
1000
800
600
400
200
0
JAK3 wild type
JAK3 M511I
thymus weight
P = .0832
Weight (mg)
300
200
100
0
JAK3 wild type
JAK3 M511I
D
spleen
bone marrow
H&E
thymus
lymph node
H&E
TdT
E
bone marrow JAK3 M511I
type I
82.8
0.7
16.4
0.1
CD8
CD4
type II
22.7
0.3
75.2
1.8
CD8
CD4

Figure 7


A
primary transplant
6 days treatment
WBC count (/mm3)
5x10^4
4x10^4
3x10^4
2x10^4
1x10^4
0
start
end
82
88
95
Days post transplant
B
primary transplant
5 weeks treatment
P = .0043
Change of WBC count (/mm3)
10000
7500
5000
2500
0
-2500
-5000
tofacitinib (n=5)
placebo (n=6)
C
secondary transplant
P = .0556
Change of WBC count (/mm3)
3.0x10^5
2.5x10^5
2.0x10^5
1.5x10^5
1.0x10^5
0.5x10^5
0
tofacitinib (n=5)
placebo (n=5)
D
P = .0159
Spleen weight (mg)
1500
1000
500
0
tofacitinib
placebo
P = .0873
Thymus weight (mg)
150
100
50
0
tofacitinib
placebo
E
spleen
thymus
placebo
tofacitinib
cleaved caspase 3
H&E