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Abstract

JAK1 and JAK3 are recurrently mutated in acute lymphoblastic leukemia. These tyrosine kinases associate with heterodimeric cytokine receptors such as IL-7R or IL-9R, in which JAK1 is appended to the specific chain and JAK3 to the common gamma chain. Here, we studied the role of these receptor complexes in mediating the oncogenic activity of JAK3 mutants. While JAK3(V674A) and the majority of other JAK3 mutants needed to bind to a functional cytokine receptor complex in order to constitutively activate STAT5, JAK3(L857P) was unexpectedly found to not depend on such receptor complexes for its activity, which was induced without receptor or JAK1 co-expression. Introducing a mutation in the FERM domain that abolished JAK-receptor interaction did not affect JAK3(L857P) activity, while it inhibited the other receptor-dependent mutants. The same cytokine receptor independence as for JAK3(L857P) was observed for homologous L(857) mutations of JAK1 and JAK2 and for JAK3(L875H). This different...

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Distinct acute lymphoblastic leukemia (ALL)-associated Janus Kinase 3 (JAK3) mutants exhibit different cytokine-receptor requirements and JAK-inhibitor specificities

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Running title: *Receptor-independent activating JAK3 mutants*

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Keywords: Janus kinase (JAK); tyrosine-protein kinase (tyrosine kinase); leukemia; oncogene; signal transduction; JAK-inhibitor

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Background: JAK3, a tyrosine kinase associated to cytokine receptors, is frequently mutated in leukemia.
Results: JAK3^{L857P} induces constitutive signaling independently of cytokine receptors and JAK1, in contrast to other JAK mutants
Conclusion: Different JAK mutants signal through distinct mechanisms and show different sensitivity to JAK1- or JAK3-specific inhibitors
Significance: Depending on the JAK residue mutated, patients will require different treatments

ABSTRACT

JAK1 and JAK3 are recurrently mutated in acute lymphoblastic leukemia. These tyrosine kinases associate with heterodimeric cytokine receptors such as IL-7R or IL-9R, in which JAK1 is appended to the specific chain and JAK3 to the common gamma chain. Here, we studied the role of these receptor complexes in mediating the oncogenic activity of JAK3 mutants. While JAK3^{V674A} and the majority of other JAK3 mutants needed to bind to a functional cytokine receptor complex in order to constitutively activate STAT5, JAK3^{L857P} was unexpectedly found to not depend on such receptor complexes for its activity, which was induced without receptor or JAK1 co-expression. Introducing a mutation in the FERM domain that abolished JAK-receptor interaction did not affect JAK3^{L857P} activity, while it inhibited the other receptor-dependent mutants. The same cytokine receptor independence as for JAK3^{L857P} was observed for homologous L^{857} mutations of JAK1 and JAK2 and for JAK3^{L875H}.

This different cytokine receptor requirement correlated with different functional properties *in vivo* and with distinct sensitivity to JAK inhibitors. Transduction of murine hematopoietic cells with JAK3^{V674A} led homogenously to lymphoblastic leukemias in BALB/c mice. In contrast, transduction with JAK3^{L857P} induced various types of lymphoid and myeloid leukemias. Moreover, Ruxolitinib, which preferentially blocks JAK1 and JAK2, abolished the proliferation of cells transformed by the receptor-dependent JAK3^{V674A}, yet proved much less potent on cells expressing JAK3^{L857P}. These particular cells were, in contrast, more sensitive to JAK3-specific inhibitors. Altogether, our results showed that different JAK3 mutations induce constitutive activation through distinct mechanisms, pointing to specific therapeutic perspectives.

JAK3 is a member of the Janus kinase (JAK) family, which comprises three other tyrosine kinases: JAK1, JAK2, and TYK2. These intracellular kinases are associated with a wide variety of cytokine receptors that lack intrinsic kinase activity. While the three other JAKs are ubiquitously expressed and can bind various receptor chains, JAK3 is exclusively associated with the common gamma chain (γc), and both genes are preferentially expressed in hematopoietic cells. γc partners with a specific alpha (or beta) chain that binds JAK1 to form the heterodimeric receptor complexes for IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 cytokines, key players of lymphoid development. For the IL-2 receptor complex (IL-2R), IL-2 stimulation revealed JAK1 to be responsible for the phosphorylation of JAK3 and signal transducers and activators of transcription 5 (STAT5), with the role of JAK3 being to phosphorylate JAK1(1).

The JAKs possess three functional domains that are well conserved. The kinase domain is the catalytic domain of the tyrosine kinases, located at the carboxyl-terminal end (C-terminus). Adjacent to this is the pseudokinase domain, which presents a kinase-like sequence, yet lacks critical residues required for proper catalytic activity. This domain is supposed to prevent inappropriate activation of the kinase domain in the absence of cytokine stimulation (2). The amino(N)-terminus contains the FERM (band-4.1, ezrin, radixin, moesin) domain. This domain mediates direct JAK interaction with the intracellular portion of cytokine receptors (3). Mutations disturbing the structural integrity of the extreme N-terminus of the FERM domain are interfering with γc association, as illustrated e.g. by the Y100C mutation of JAK3 (4).

The JAK3^{Y100C} mutation was described in patients suffering from severe combined immunodeficiency (SCID) (5). This germline JAK3 inactivating mutation abrogates the γc-dependent cytokine signaling essential for lymphoid cell development, leading to a profound defect in T, B, and NK cells. In contrast to these inactivating mutations, acquired JAK3 activating point mutations have been described in various leukemia types, such as T-cell acute lymphoblastic leukemia (T-ALL) (6), acute megakaryoblastic leukemia (AMKL) (7) or juvenile myelomonocytic leukemia (JMML) (8) resulting in constitutive JAK activation and STAT phosphorylation.

The severe effect of JAK3 signaling impairment specifically impacting on lymphoid development in SCID patients has instigated the development of immunosuppressive drugs targeting this kinase. Tofacitinib, a type-I JAK inhibitor targeting JAK3 (1), is currently an approved treatment for rheumatoid arthritis in the United States. This drug is being assessed for use in the treatment of T-cell specific autoimmune diseases, such as psoriasis, multiple sclerosis, and inflammatory bowel disease, as well as for the prevention of organ transplant rejection (9,10). While originally described as a selective JAK3 inhibitor, tofacitinib also inhibits JAK1, and its efficacy is likely due to combined inhibition of both kinases (1,11). On the other hand, JAK inhibitors are also currently being investigated for the treatment of hematological malignancies caused by abnormal JAK activation. Ruxolitinib, a JAK1/2 type-I inhibitor, is currently administered in the treatment for myeloproliferative neoplasms (MPNs), which are frequently associated with the V617F activating mutation in JAK2 (12). In line with the results observed with JAK inhibitor use in MPN treatment, these inhibitors represent an appealing therapeutic strategy for leukemia cases associated with JAK3 activation.

This study sought to gain more insight into the potential use of JAK inhibitors in mutated JAK3-driven malignancies. We therefore decided to first determine if ALL-associated JAK3 mutants need to bind γc in order to be constitutively active, then to test the sensitivity of different mutants to JAK inhibitors.

EXPERIMENTAL PROCEDURES

Cells, cell culture and cytokines- Ba/F3 pro-B cells were cultured in Iscove-Dulbecco's medium supplemented with 10% fetal bovine serum, 50μM ß-mercaptoethanol, 0.55 mM L-arginine, 0.24 mM L-asparagine, 1.25 mM L-glutamine and in the presence of murine IL-3. Autonomous Ba/F3 cells expressing JAK3 mutants or BCR-ABL were cultured without IL-3. BCR-ABL transduced Ba/F3 cells were a kind gift of Jean-Baptiste Demoulin (Université catholique de Louvain, Brussels, Belgium). HEK293 human embryonic kidney cells and JAK1 deficient U4C cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. These cells do not express endogenous IL-9Rα, γc and JAK3.

Plasmid constructions- Human (h)JAK3 mutants and double mutants cDNA's (Y100A, M511I, A572V, V674A, V674A/Y100A, L857P, L857A, L857E and L857P/Y100A), murine (m)JAK1 mutants and double mutants (V658F, K907A as kinase-dead mutation, L910P and L910P/Y107A), and mJAK2 mutants and double mutants (V617F, L884P and L884P/Y114A) were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene, La Jolla, California) and subcloned into the pMX-IRES-CD4 (JAK3) or pMX-IRES-GFP (JAK1 and JAK2) biscistronic retroviral vectors upstream of the IRES. The WT, V674A, L857Q, L875H, P906S and E958K hJAK3 pMSCV-GFP plasmids were described earlier (13). Mutagenesis was performed on the WT plasmid to create Y824A, T848A, L857P and L857P/Y100A hJAK3 mutants. WT hJAK3 and hIL-9 receptor α cDNA were subcloned into the pMX-IRES-CD4 biscistronic retroviral vector. WT mJAK1, WT mJAK2 and human common γ chain cDNA were subcloned into the pMX-IRES-GFP biscistronic retroviral vector.

Virus production, stable DNA transfections, analysis of transfected cells- Retroviral supernatants were generated by transient transfection of HEK293T cells with the packaging plasmid pCL-Eco (coding for gag/pol/env) and biscistronic vectors encoding the gene of interest and human CD4 as marker, and used for infection of 0.5 x 10^6 Ba/F3 cells. HEK293 cells were transfected with 3.75 µg of pCL-Eco and 3.75 µg of the different constructs with 22.5 µl of TransIT-LT1 Transfection Reagent (Mirus). The next day, supernatants were collected and added to Ba/F3 cells for the spin-infection (two hours incubation at 1000g at room temperature). This last step was repeated the day after to increase the transfection level. Expression of the markers was analyzed by FACS (BD FACSCalibur^{TM} flow cytometer), using a PE-coupled antibody against hCD4 (#555347, BD Pharmingen) diluted 1/30. For murine bone marrow cells transduction, mutated JAK3 pMSCV-GFP viral vectors were produced in HEK293T cells using an EcoPack packaging plasmid and TurboFect transfection reagent (Fermentas). Virus was harvested after 48 hours.

Dual luciferase assay- STAT5 transcriptional activity was assessed by measurements of luciferase expression in HEK293 cells upon transient transfection of appropriate cDNA constructs and pLHRE-luc vector harboring tandem copies of the STAT5-inducible lactogenic hormone response element (LHRE) of the rat β-casein promoter, inserted upstream a luciferase gene (14). Another reporter plasmid, *Renilla* luciferase (pRLTk, Promega), was co-transfected as an internal transfection control. Transient transfection of HEK293 cells by LipofectAMINE (Invitrogen) was previously described (15). 24 hours after transfection, luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega).

Western blots- For Western Blot analysis of JAK3 expression in HEK293 cells, 4 x 10^5 cells were transfected with 1 µg of different JAK3 constructs with LipofectAMINE (Invitrogen) as previously described (15). Cell lysate preparation, gel electrophoresis and transfer to nitrocellulose membranes were performed as previously described (16). Phosphorylation of signaling proteins was investigated with following phospho-specific antibodies from Cell Signaling Technology: anti-pY1034/1035 JAK1 (#3331), anti-pY980/981 JAK3 (#5031), anti-pY705 STAT3 (#9131), anti-pY694 STAT5 (#9351) and anti-pT202/Y204 ERK1/2 (#9101). Blots were re-probed with anti-JAK1 (#3332), anti-JAK3 (#3775), anti-STAT3 (#9132) (Cell Signaling Technology, Beverly, MA), anti-STAT5 (#SC-835, Santa Cruz Biotechnology) or anti-β-actin (#A5441, Sigma) antibodies, as a control.

Murine bone marrow transplantation- BALB/c mice were purchased from Charles River Laboratories. Harvest of bone marrow cells from male donor mice, lineage negative cells enrichment, transduction with viral supernatant and injection into irradiated female recipient mice was performed as previously described (13). After sacrifice of the mice, single-cell suspensions were prepared from peripheral blood, bone marrow, spleen, thymus and lymph nodes. Cells were analyzed on a FACSCanto flow cytometer (BD Bio-sciences).

Proliferation assays- For knockdown experiments, 1.5 × 10^6 IL-3 dependent control Ba/F3 cells or growth factor-independent Ba/F3 cells obtained after transduction with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) were resuspended in 400 µL of medium containing 200 nM siRNA duplexes (Silencer Select Il2rg Mouse, Ambion cat#s68269 ; Stealth siRNA Jak1 Mouse, Invitrogen cat#mss205625 ; Silencer Select Negative Control No. 1 siRNA, Ambion cat#4390843 ; Stealth RNAi™ siRNA Negative Control, Invitrogen cat#12935-400) and then transferred to 4-mm cuvettes (Biorad). Cells were electroporated (260V, 90Ω, 1500µF) and seeded in 96-well plates at 10,000 cells/well. For JAK inhibitors treatment, Ba/F3 cells stably transduced with ALL-associated JAK3 mutants (V674A and L857P), double mutant (L857P/Y100A) or BCR-ABL were seeded in 96-well plates at 5,000 cells/well in the presence of increasing concentrations of Ruxolitinib (INCB018424, Haoyuan Chemexpress Co), NIBR3048 (Tocris Bioscience (TSC21311), (17)) or Tofacitinib (CP-690550, Selleckchem). After 72 hours, methyl-H^3Thymidine (Amersham, cat#TRK120) was added to the cells for 4 hours, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT).

JAK3 structure- The three-dimensional structural model of the pseudokinase domain of JAK3 was obtained by employing the SWISS-MODEL server and was superimposed on TYK2 JH1-JH2 crystal structure (PDB code: 4OLI) as template, together with the JAK3 kinase domain crystal structure (PDB code: 1YVJ), using the Pymol program.

RESULTS

ALL-associated JAK3 mutants signal through a functional cytokine receptor complex, except the L857P mutant- More and more patients suffering from different types of leukemia, such as ALL, AMKL or JMML, have been reported to exhibit mutations in the JAK3 gene (6-8,18,19). These mutations were often characterized as activating (7,18-20), yet little is known about their activation mechanism. Of the other JAK kinases, JAK2 V617F, found in 95% of polycythemia vera cases, has been shown to be dependent on homodimeric receptors, like the erythropoietin receptor (EpoR), in order to become active (20). Similar results were obtained for the activating JAK1 V658F and A634D mutants, which needed to bind to IL-9 receptor alpha or IL-2 receptor beta in order to form homo- or heterodimeric cytokine receptor complexes able to mediate constitutive signaling (21). Furthermore, the active $\text{JAK3}^{\text{A572V}}$ mutant requires heterodimeric complexes formed by IL-9 receptor alpha or IL-2 receptor beta and γc (22). We sought to determine if ALL-associated JAK3 mutants shared similar receptor-dependent mechanisms of constitutive signaling. To this end, we studied four activating JAK3 mutants scattered throughout the kinase and pseudokinase domains, namely M511I, A572V, V674A, and L857P identified in patients with T-ALL (Marco Tartaglia, unpublished data). We took advantage of HEK293 cells that are deficient in JAK3 and γc, the only cytokine receptor chain known to bind JAK3. WT JAK3 and the four mutants were transiently transfected into HEK293 cells, in combination with a STAT5-responsive reporter construct. This transfection was carried out both in the absence of IL-9Rα or γc, or in their presence in order to reconstitute a functional IL-9R complex. As shown in figure 1, the four tested JAK3 mutants induced constitutive STAT5 activation, although for the M511I, A572V, and V674A mutants, this was achieved only in the presence of a functional IL-9R complex (γc and IL-9Rα). Surprisingly, JAK3^{L857P} induced STAT5 activation even without co-expression of any receptor chain. This observation suggested a receptor-independent activity that was specific to JAK3^{L857P}. An alternative hypothesis would be the involvement of another cytokine receptor chain that could bind JAK3 and would be expressed in HEK293. To exclude the latter hypothesis, we tested the effect of a SCID mutation localized in the FERM domain of JAK3 (Y100C) that inactivates the JAK by disabling its binding to γc (4,5). In fact, mutation of this conserved hydrophobic residue in JAK FERM domains disturbs the structural integrity of parts of this FERM domain which plays an essential role in all JAK-cytokine receptor interactions, as also shown for the homologous mutations of JAK1 (Y107A) (3) and JAK2 (Y114A) (23). We introduced the Y100A mutation into a WT, V674A and L857P JAK3 and evaluated the capacity of each mutant and double mutant to mediate constitutive signaling. As illustrated in figure 2A, the JAK3^{V674A} activity in the presence of the IL-9R complex was abrogated by introducing the Y100A mutation, thereby confirming the essential role of Y100 for receptor binding. This observation contrasted with the results obtained for JAK3^{L857P} for which introducing the Y100A mutation did not abrogate its activity. On the contrary, increased constitutive signaling in the absence of a functional receptor was observed. Notably, the expression level of each JAK3 mutant was similar under all experimental conditions (figure 2B).

To confirm the lack of γc requirement for constitutive JAK3^{L857P} signaling in hematopoietic cells, we downregulated the expression of endogenous murine γc in growth factor-independent Ba/F3 cells obtained after stable transduction with JAK3^{V674A}, JAK3^{L857P}, and JAK3$^{L857P/Y100A}$, or in IL-3 dependent control Ba/F3 cells. Figure 2C shows that siRNA-mediated knockdown of γc resulted in a significant decrease of the proliferation of Ba/F3 cells transformed with JAK3^{V674A} compared to the proliferation observed after control siRNA treatment, while no decrease in proliferation was observed for cells transformed by JAK3^{L857P} or JAK3$^{L857P/Y100A}$. These results confirm the unique capacity of JAK3^{L857P} to induce cytokine-receptor independent constitutive signaling.

Cytokine receptor complexes are formed of two receptor chains, each associated with one JAK. In γc-associated receptor complexes, such as IL-7 or IL-9 receptor, JAK3 and JAK1 are believed to interact and cross-phosphorylate each other to enable signaling. In order to assess the role of JAK1 in mutant JAK3-induced signaling, we took advantage of JAK1-deficient U4C cells. As shown in figure 3A, in the absence of JAK1, the JAK3^{V674A} was unable to activate STAT5, even when cytokine receptor chains were expressed. This activity was restored by co-expression with JAK1^{WT}, but not with a kinase-dead (KD) JAK1. The results obtained for JAK3^{L857P}, shown in figure 3B, indicated that the activity of this mutant is entirely independent of JAK1, once again supporting the hypothesis of a cytokine receptor-independent activation mode.

To confirm the lack of JAK1 requirement for constitutive JAK3^{L857P} signaling in hematopoietic cells, endogenous JAK1 knockdown was performed as described for γc (figure 3C). This knockdown decreased the proliferation of Ba/F3 cells transformed with JAK3^{V674A}, while it did not alter the proliferation of cells transformed by JAK3^{L857P} or JAK3$^{L857P/Y100A}$. Taken together, these results revealed JAK3^{L857P} as possessing a unique characteristic: the capacity to activate signaling in the absence of binding to a cognate cytokine receptor complex. This contrasts with the other tested JAK3 mutants as well as all JAK1 and JAK2 activating mutants described in the literature (20,21).

Given that the leucine 857 of JAK3 was conserved among all JAK kinases, we sought to determine if mutations of the homologous residue in JAK1 (L910) and JAK2 (L884) could confer a similar receptor-independent constitutive signaling capacity. Those mutations were introduced together with the FERM domain mutations Y107A in JAK1, or

Y114A in JAK2 (homologous to Y100A in JAK3). As illustrated in figure 4, upon transient expression in HEK293 cells, $JAK1^{L910P/Y107A}$ and $JAK2^{L884P/Y114A}$ were capable of activating STAT5, demonstrating that the activating mutations of a leucine residue conserved among JAK kinases share the capacity of inducing STAT5 activation independently of the receptor-FERM domain interaction.

To gain more insight about potential mechanisms enabling receptor-independent constitutive activation of $JAK3^{L857P}$, we substituted L857 with different residues and tested the receptor-independent activity of these JAK3 mutants in HEK293 cells, using JAK3 cloned in either pMX-IRES-CD4 (Figure 5A) or pMSCV-GFP (Figure 5B). Receptor-independent constitutive STAT5 activation was observed after replacing the leucine with a proline, alanine or glutamine, but not with a glutamic acid, probably due to this residue's negative charge, even if the mutant was still active in presence of IL-9, (*data not shown*).

Another kinase domain mutant, $JAK3^{L875H}$, exhibits the same receptor-independent activating capacity as $JAK3^{L857P}$- Among the JAK3 mutants studied here, L857P was the only mutant involving the kinase domain, raising the hypothesis that cytokine receptor independence could be explained solely by the mutation's location in the kinase domain, compared to the pseudokinase mutations that would be receptor-dependent. To address this question, other activating mutations of the kinase domain were investigated. These consisted of one JAK3 mutation associated with T-cell pro-lymphocytic leukemia (Y824D; (24)), three ALL-associated mutants (L875H, P906S, E958K; Jan Cools, unpublished data), and one mutant described in a murine T-cell line (T848A;(16)). As illustrated in figure 6A, four out of these five new mutants were dependent on the co-expression of IL-9Rα and γc for their activity in HEK293 cells. In contrast, $JAK3^{L875H}$ behaved like $JAK3^{L857P}$, exhibiting a receptor-independent activating capacity. The close proximity of L857P and L875H mutants to the α-helix C of the kinase domain is illustrated in the predicted protein structure of JAK3 kinase domain, represented in figure 6B. By contrast, the receptor-dependent mutants map to the interface between pseudokinase and kinase domains, as previously described (16).

The difference in cytokine-receptor requirement correlates to a distinct JAK inhibitor specificity- Given that $JAK3^{L857P}$ and the other JAK3 mutants presented different receptor and JAK1 requirements, we sought to determine if they would exhibit the same sensitivity to ruxolitinib, a JAK1-2 inhibitor. In order to assess this, we treated growth factor-independent Ba/F3 cells obtained after stable transduction with $JAK3^{V674A}$, $JAK3^{L857P}$, and $JAK3^{L857P/Y100A}$, with increasing ruxolitinib concentrations and measured their proliferation using a tritiated thymidine incorporation assay. We employed Ba/F3 cells that had been stably transduced with the chronic myeloid leukemia-associated BCR-ABL fusion gene as a negative control, choosing this particular oncogene for its lack of sensitivity to JAK inhibitors. Figure 7A illustrates that Ba/F3 cells transformed with $JAK3^{V674A}$ were at least ten fold more sensitive to ruxolitinib than those transformed with $JAK3^{L857P}$ and $JAK3^{L857P/Y100A}$. The calculated IC50 are shown in Table 1. This difference in ruxolitinib sensitivity between the V674A and L857P/Y100A JAK3 mutants was reproduced in transiently transfected HEK293 cells treated with 0.5μM of ruxolitinib, STAT5 transcriptional activity being used as readout (figure 7B). To complement these observations, we monitored the phosphorylation status of STAT5, JAK1, and JAK3 in growth factor-independent $JAK3^{V674A}$ and $JAK3^{L857P/Y100A}$ Ba/F3 cells treated with 0.5, 1, and 2μM of ruxolitinib (figure 7C). This JAK1-2 inhibitor abrogated STAT5 phosphorylation induced by the V674A mutant, whereas it had a marginal effect on the L857P/Y100A double mutant, thus confirming the difference in ruxolitinib sensitivity. While able to inhibit STAT5 phosphorylation by $JAK3^{V674A}$, ruxolitinib did not inhibit JAK1 phosphorylation, probably because it is a type I kinase inhibitor, blocking the kinase in an active conformation that can still be phosphorylated by JAK3. JAK3 phosphorylation was only observed in the double mutant cells, with no ruxolitinib-induced inhibition observed, in line with the absence of effect seen with STAT5. Altogether, these results demonstrate that the activity of JAK3^{V674A} can be blocked with a JAK1-specific inhibitor, while JAK1-independent JAK3^{L857P} is much less sensitive to ruxolitinib.

We next tested the effect of a JAK3-specific inhibitor (NIBR3049; (17)) on the different JAK3 mutants by performing the same experiments as for ruxolitinib. As illustrated in figure 8A, the Ba/F3 cells transformed with JAK3^{L857P} and JAK3$^{L857P/Y100A}$ proved more sensitive to NIBR3049 than those transformed with JAK3^{V674A}. The calculated IC50 are shown in Table 1. Similar results were obtained for STAT5 activity in HEK293 cells (figure 8B). In Ba/F3 cells, the NIBR3049 inhibitor completely blocked STAT5 and JAK3 phosphorylation in the JAK3$^{L857P/Y100A}$ cells, confirming their sensitivity to this molecule (figure 8C). By contrast, NIBR3049 barely affected STAT5 and JAK1 phophorylation in JAK3^{V674A} cells and even increased JAK3 phosphorylation because this type I inhibitor blocked JAK3 in the active conformation without affecting the kinase activity of the JAK1 partner. Altogether, these results show that JAK3^{L857P} activity can be blocked by a JAK3-specific inhibitor, whereas the JAK3^{V674A} is much less sensitive to NIBR3049. Finally, when an inhibitor active against both JAK1 and JAK3 was used (tofacitinib), both receptor-dependent and -independent mutants exhibited a similar sensitivity, as illustrated by a decrease in the proliferation of Ba/F3 cells and in STAT5 activity in HEK293 cells (figure 9).

JAK3 mutants cause distinct leukemia phenotypes in a murine hematopoietic stem cell transplant model- Degryse *et al*. reported that mice transplanted with bone marrow progenitor cells expressing T-ALL-associated JAK3 mutants developed a disease resembling T-ALL, characterized by an accumulation of immature CD8 positive T-cells (13). This phenotype can be explained as the result of constitutive activation of γc-associated receptors, which are essential for T-cell lymphoproliferation. Given that JAK3^{L857P} and JAK3$^{L857P/Y100A}$ exhibit no cytokine receptor restriction *in vitro*, we wondered if their expression in hematopoietic stem cells would be leukemogenic and produce the same phenotype *in vivo*. Hematopoietic progenitor cells were transduced with retroviral vectors expressing JAK3^{V674A}, JAK3^{L857P}, or JAK3$^{L857P/Y100A}$, together with green fluorescent protein (GFP), and injected into irradiated Balb/c mice. As shown in table 2, almost all the mice exhibited increased white blood cell (WBC) counts ranging from 20,000 to 300,000 cells/microliter at sacrifice, within 50 to 200 days post-transplant. JAK3^{V674A} mice displayed an accumulation of CD8 single positive cells in the spleen and bone marrow (table 2A and figure 10A), as well as in the peripheral blood, thymus, lymph nodes (data not shown). These mice homogenously developed a disease resembling T-ALL, similar to the one described by Degryse *et al*. By contrast, JAK3^{L857P} and JAK3$^{L857P/Y100A}$ were found to induce at least four different types of lympho- or myeloproliferative diseases, characterized by an accumulation of either CD4/8 double positive T-lymphocytes (table 2B and figure 10B), Gr1 positive myeloid cells (table 2C and figure 10C), CD8 single positive T-lymphocytes (table 2D and figure 10D), or B220 positive B-lymphocytes (table 2E and figure 10E). These results indicated that JAK3^{L857P} and JAK3$^{L857P/Y100A}$ exhibit an oncogenic potential *in vivo,* but induced a more heterogeneous phenotype compared to cytokine receptor-restricted JAK mutants.

DISCUSSION

The originality of our observations resides in the demonstration that cancer-associated activating mutations of different residues in the same JAK kinase induce different functional properties, both *in vitro* and *in vivo*. *In vitro*, different ALL-associated JAK3 mutants were associated with distinct sensitivity to JAK inhibitors, while the *in vivo* transduction of these mutants in hematopoietic precursors produced distinct leukemia phenotypes. This can, for the most part, be explained by the unexpected behavior of JAK3^{L857P}, which did not require cytokine receptor binding in order to be active. These findings contrast sharply with what has previously been published about other cancer-associated JAK mutants (20,21), including JAK3^{V674A}, which required binding to a functional receptor complex in order to be active.

The different phenotypes observed *in vivo* can also be explained by the different cytokine receptor requirements of the various mutations tested. The transduction of murine hematopoietic cells with JAK3^{V674A} led to homogenous lymphoblastic leukemias. As the oncogenic activity of this mutant is restricted to cells expressing γc, downstream constitutive signaling mimics the signaling induced by γc-associated receptors like IL-2R, IL-7R or IL-9R, which are essential for lymphoproliferation. In contrast, transduction with JAK3^{L857P} induced various types of lymphoid and myeloid leukemias in mice, revealing the existence of a broader oncogenic potential resulting from the absence of cytokine receptor restriction.

The second key observation reported here is that different activating mutations of JAK3 dictate specific pattern of sensitivity to JAK inhibitors. Such different sensitivities to JAK inhibitors could be explained by the model presented in figure 11. Cytokine receptor complexes are formed of two chains, each associated with one JAK, namely JAK1 and JAK3 for the γc cytokine receptor family (figure 11A). JAK3^{L857P}, while still able to bind to γc, does not require this binding to be active. This explains its JAK1 independent activity and accounts for the inefficacy of JAK1 inhibitors, such as ruxolitinib (figure 11B). In contrast, a JAK1 inhibitor blocks the activity of classical JAK3 mutants, such as JAK3^{V674A}, since these mutants are dependent on a functional cytokine receptor complex for signaling. A JAK3-specific inhibitor, such as NIBR3049, effectively inhibits the activity of JAK3^{L857P} (figure 11C). Its relative inefficacy against JAK3^{V657A} is in line with the role of the kinase activity of JAK3 in the context of IL-2 signaling, as reported by Haan *et al.* (1). Indeed, these authors demonstrated that although signal transduction by the IL-2R complex required JAK3 expression, it was not abrogated by a kinase-defective JAK3, suggesting that JAK1 kinase activity was sufficient for the complex to be active, with JAK3 serving as a scaffold protein. One hypothesis is therefore that classical mutants, such as JAK3^{V674A}, modify the conformation of JAK3 as a scaffold in the receptor complex in order to enable JAK1 constitutive kinase activity.

Our findings provide further evidence that although up to 30% of ALL cases might harbor activating mutations of the IL-7R-JAK1-JAK3 pathway, working with JAK inhibitors to treat such hematological neoplasms will not be a simple task. The selection and dosing of the inhibitor must not only be based on the kinase affected, but also on the specific mutation observed. We previously showed that activating mutations affecting Phe958 and Pro960 of JAK1 not only promoted autonomous cell proliferation, but also conferred resistance to ATP-competitive inhibitors, suggesting that mutations of the same kinase could require different JAK inhibitor dosages (25). This also proved true for mutations of the JAK2 Tyr931 residue (25) and, furthermore, applied to the homologous mutation of the Tyr904 residue of JAK3 (16). Our current study has demonstrated that different JAK inhibitors are required to block different JAK3 mutants, depending on their mechanisms of signal transduction activation. While JAK1 (L910P) and JAK2 (L884P) mutations that are homologous to L857P also induce cytokine receptor-independent constitutive signaling, their effect on JAK inhibitor sensitivity should be less critical, as many JAK2-associated receptors are homodimeric and the kinase activity of JAK1 appears essential for γc-associated receptors.

The mechanisms enabling the receptor-independent constitutive activation of JAK3^{L857P} are still subject to speculation. For JAK3, receptor-independent constitutive activation is observed after replacing the leucine with different residues (figure 5), except for glutamic acid. JAK3^{L857Q} has been described in ALL patients (13), and is also receptor-independent. The L910Q mutation of JAK1 has already been described as activating in a random mutagenesis screening analysis (26), and introducing the Y107A mutation (homologous to the JAK3 Y100A mutation) in the FERM domain of JAK1^{L910Q} has been found to not completely abolish STAT5 constitutive phosphorylation. The diversity of these residues suggests that the loss of leucine 857 is the key factor inducing constitutive activation. Interestingly, the leucine 857 of JAK3 is not only conserved among all JAK kinases, but also in other tyrosine kinases, such as c-Src, LCK, c-ABL, EGFR, FGFR, and PDGFR. The relevance of this residue for kinase activity regulation is illustrated by the EGFR exon 19 insertions leading to the L747P mutation described in patients with lung adenocarcinoma, or by the L747S mutation of the EGFR, reported in patients acquiring secondary resistance to EGFR inhibitors. It is believed to shift the equilibrium towards the receptor's active conformation (27-29). Other arguments point towards the importance of the JAK αC helix in the regulation of the cytokine receptor dependence. As illustrated in 4B, the L857 residue is situated in front of the αC helix of the N-lobe of the JAK3 kinase domain. This alpha helix is conserved among all kinases and constitutes a key mediator of conformational change between the kinase's active and inactive state (30). Additionally, another mutation located within the αC helix of JAK3, L875H, is present in patients with ALL, exhibiting the same characteristics as L857P, namely constitutive activation and receptor independence.

A further puzzling observation is that when the Y100A mutation was added to the active JAK3^{L857P} mutant, STAT5 activation was seen to increase in HEK293 cells. A preliminary explanation for this could be that Y100A increases L857P-induced constitutive kinase activity. Previous studies have, in fact, suggested that the FERM domain fulfills a regulatory role in kinase activity through interaction between the kinase and FERM domains (2,31,32) Moreover, electron microscopy JAK1 imaging revealed this protein as exhibiting a high flexibility, able to shuttle from open to closed states (32). In the compact conformation, the FERM domain comes in close proximity to the kinase domain. The Y100A mutation could change these interactions and increase the kinase activity induced by L857P. Another explanation could reside in the subcellular location of the kinase. JAK3^{V674A} needs to bind to a cytokine receptor in order to be active, with a kinase activity located close to the plasma membrane. The localization of JAK3$^{L857P/\ Y100A}$ is unknown, yet cytokine-receptor independent. The constitutive kinase activity could therefore take place in a location where the activation of downstream signaling is more efficient, such as the cytoplasm. Further studies will be needed to address these issues

Because of their particular mode of action, the cytokine receptor-independent active mutants of JAK kinases described here open new perspectives, not only for treating patients with alterations of the JAK-STAT pathway, but also for a better understanding of this pathway.

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Author contributions:
LK, JCR, EL, TK and LS designed the study. EL, JCR and LK wrote the paper. EL, SD and OG performed the experiments. All authors analyzed the results and approved the final version of the manuscript.

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FOOTNOTES

The abbreviations used are: ALL, Acute Lymphoblastic Leukemia; AMKL, Acute Megakaryoblastic Leukemia; γc, Common Gamma Chain; EpoR, Erythropoietin Receptor; GFP, Green Fluorescent Protein; IL, Interleukine; IL-2R, Interleukine 2 receptor; IL-9R, Interleukine 9 receptor; IL-9Rα, Alpha Chain of the Interleukine 9 Receptor; JAK, Janus Kinase; JMML, Juvenile Myelomonocytic Leukemia; KD, Kinase-Dead; LHRE, Lactogenic Hormone Response Element MPN, Myeloproliferative neoplasm; SCID, Severe Combined Immunodeficiency Syndrome; STAT, Signal Transducer and Activator of Transcription; WBC, white blood cells

FIGURE 1. *STAT5 activation by JAK3 activating mutants is receptor-dependent, except for the L857P mutant-* JAK3- and γc-deficient HEK293 cells were transiently co-transfected with JAK3^{WT}, or ALL-associated A572V, V674A, M511I and L857P JAK3 mutants, and γc and/or IL-9Rα, in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of triplicates. Similar results were obtained in 3 independent experiments. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK3 and the WT or mutant forms of JAK3 for each condition (with or without IL-9R complex) ($^*p<0.05$, $^{**}p<0.01$).

FIGURE 2. *JAK3 FERM domain integrity is required for the activity of the V674A mutant, but not for the L857P mutant-* **(A)** HEK293 cells were transiently co-transfected either with JAK3^{WT}, or different JAK3 mutants (Y100A, V674A, L857P), or double mutants (V674A/Y100A, L857P/Y100A) together with γc and IL-9Rα or not, in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK3 and the WT or mutant forms of JAK3 for each condition (with or without IL-9R complex) ($^*p<0.05$, $^{***}p<0.001$). K = kinase domain, PK = pseudokinase domain. **(B)** In parallel of the luciferase assay, transfected HEK293 cells were lyzed 24 hours post-transfection and subjected to Western Blot analysis using an anti-JAK3 antibody and an anti-β-Actin antibody as loading control. **(C)** Relative proliferation of IL-3 dependent Ba/F3 cells or autonomous Ba/F3 cells obtained after transduction with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) after knockdown of endogenous γc compared to the proliferation observed with an irrelevant control siRNA. After 72 hours, tritiated thymidine incorporation was measured. Results are mean ± standard deviation of three different experiments each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the IL-3 dependent control Ba/F3 cells and the transformed Ba/F3 cells ($^*p<0.05$).

FIGURE 3. *JAK1 expression s required for the activity of the V674A mutant, but not for the L857P mutant-* **(A)** JAK1-deficient U4C cells were transiently co-transfected with JAK3^{WT} or $\text{JAK3}^{\text{V674A}}$, γc and IL-9Rα with or without JAK1^{WT} or JAK1^{KD}, in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values ($^*p<0.05$) **(B)** JAK1-deficient U4C cells were transiently co-transfected with JAK3^{WT} or $\text{JAK3}^{\text{L857P}}$, γc and IL-9Rα with or without JAK1^{WT} or JAK1^{KD}, in addition to the STAT5-responsive luciferase reporter pLHRE-luc and the pRLTK plasmid as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values ($^*p<0.05$). **(C)** Relative proliferation of IL-3 dependent Ba/F3 cells or autonomous Ba/F3 cells

obtained after transduction with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) after knockdown of endogenous JAK1 compared to the proliferation observed with an irrelevant control siRNA. After 72 hours, tritiated thymidine incorporation was measured. Results are mean ± standard deviation of three different experiments each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the IL-3 dependent control Ba/F3 cells and the transformed Ba/F3 cells (**$p<0.01$).

FIGURE 4. *Integrity of the FERM domain is not required for activation of JAK1 and JAK2 through homologous mutation of* $JAK3^{L857P}$- (A) HEK293 cells were transiently co-transfected with ALL-associated $JAK1^{V658F}$, $JAK1^{L910P}$ (homologous to JAK3 L857P), or $JAK1^{L910P/Y107A}$, with or without IL-9Rα, in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of means of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK1 and the mutant forms of JAK1 for each condition (with or without IL-9Rα) (*$p<0.05$, **$p<0.01$, ***$p<0.001$). (B) HEK293 cells were transiently co-transfected with MPN-associated $JAK2^{V617F}$, $JAK2^{L884P}$ (homologous to JAK3 L857P), or $JAK2^{L884P/Y114A}$, in addition to the STAT5-responsive luciferase reporter pLHRE-luc and the pRLTK plasmid as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. Results are mean ± standard deviation of means of triplicates of three different experiments. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK2 and the mutant forms of JAK2 (*$p<0.05$, ***<0.001).

FIGURE 5. *Substitution of L857 of JAK3 with different residues can confer receptor-independent constitutive activity*- HEK293 cells were transiently transfected either with wild-type JAK3, or different JAK3 mutants: **(A)** $JAK3^{WT}$, $JAK3^{V674A}$, $JAK3^{L857P}$, $JAK3^{L857A}$, $JAK3^{L857E}$ in pMX-IRES-CD4 and **(B)** $JAK3^{WT}$, $JAK3^{V674A}$, $JAK3^{L857P}$, $JAK3^{L857Q}$ in pMSCV-GFP. The STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) were co-transfected. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of means of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK3 and the WT or mutant forms of JAK3 for each condition (*$p<0.05$, **$p<0.01$, ***$p<0.001$).

FIGURE 6. *The majority of kinase domain mutants are receptor-dependent, except* $JAK3^{L857P}$ *and* $JAK3^{L875H}$- **(A)** HEK293 cells were transiently co-transfected either with $JAK3^{WT}$, or different JAK3 mutants (V674A, L857P, Y824A, T848A, L875H, P906S, E958K), in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of means of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values between the control condition without JAK3 and the WT or mutant forms of JAK3 for each condition (*$p<0.05$,

p<0.01, ***p<0.001). **(B) Localization of ALL-associated JAK3 mutants of the kinase domain. The figure represents the kinase and pseudo-kinase domains of JAK3 based on the recent TYK2 JH1/JH2 crytsal structure (33). The kinase domain is shown in indigo, with the αC helix in yellow and the activation loop in orange. The adjacent pseudokinase domain is shown in green. Mutated residues close to the pseudokinase domain are indicated with pink balls and mutated residues close to the αC helix are indicated with light-blue balls.

FIGURE 7. *Cells expressing JAK3^{V674A} mutant are more sensitive to Ruxolitinib than cells expressing JAK3^{L857P} or JAK3$^{L857P/Y100A}$*- **(A)** Autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) or BCR-ABL as a control, were treated with increasing concentrations of JAK1/JAK2 inhibitor Ruxolitinib (0-3μM). After 72 hours, tritiated thymidine incorporation was measured. Results are mean ± standard deviation of three different experiments each performed in triplicate, represented in % of the proliferation of the respective untreated cells. **(B)** HEK293 cells were transiently co-transfected either with JAK3^{V674A} or JAK3$^{L857P/Y100A}$, with a receptor complex (Rec = γc, IL-9Rα and JAK1^{WT}), in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. Cells were treated with Ruxolitinib (1μM). 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values (*p<0.05). **(C)** 10^6 autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutant V674A or double-mutant L857P/Y100A were treated with increasing concentration of Ruxolitinib (0-2μM). Two hours after treatment, cells were lyzed and subjected to Western Blot analysis. Phosphorylation of STAT5, JAK3 and JAK1 was detected using specific anti-pY694 STAT5, anti-pY980/81 JAK3, and anti-pY1034/35 JAK1 antibodies. Membranes were re-probed with anti-STAT5, anti-JAK3, anti-JAK1 and anti-β-Actin antibodies as loading controls.

FIGURE 8. *Cells expressing JAK3 L857P or L857P/Y100A mutants are more sensitive to NIBR3049 than cells expressing JAK3 V674A*- **(A)** Autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) or BCR-ABL as a control, were with increasing concentrations of JAK3 inhibitor NIBR3049 (0-2μM). After 72 hours, tritiated thymidine incorporation was measured. Results are mean ± standard deviation of three different experiments each performed in triplicate, represented in % of the proliferation of the respective untreated cells. **(B)** HEK293 cells were transiently co-transfected either with JAK3^{V674A} or JAK3$^{L857P/Y100A}$, with a receptor complex (Rec = γc, IL-9Rα and JAK1^{WT}), in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla luciferase) as transfection control. Cells were treated with NIBR3049 (0.5μM). 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values (*p<0.05). **(C)** 10^6 autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutant V674A or double-mutant L857P/Y100A were treated with increasing concentration of NIBR3049 (0-4μM). Two hours after treatment, cells were lyzed and subjected to Western Blot analysis. Phosphorylation of STAT5, JAK3 and JAK1 was detected using specific anti-pY694 STAT5, anti-pY980/81 JAK3, and anti-pY1034/35 JAK1 antibodies. Membranes were re-probed with anti-STAT5, anti-JAK3, anti-JAK1 and anti-β-Actin antibodies as loading controls.

FIGURE 9. *Cells expressing JAK3 V674A, L857P and L857P/Y100A mutants are sensitive to Tofacitinib-* (A) Autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutants (V674A and L857P) and double mutant (L857P/Y100A) or BCR-ABL as a control, were treated with increasing concentrations of JAK1/JAK3 inhibitor Tofacitinib (0-3μM). After 72 hours, tritiated thymidine incorporation was measured. Results are mean ± standard deviation of three different experiments each performed in triplicate, represented in % of the proliferation of the respective untreated cells. (B) HEK293 cells were transiently co-transfected either with JAK3^{V674A} or JAK3$^{L857P/Y100A}$, with a receptor complex (Rec= γc, IL-9Rα and JAK1^{WT}), in addition to the STAT5-responsive luciferase reporter pLHRE-luc (firefly luciferase) and the pRLTK plasmid (renilla lucifearse) as transfection control. Cells were treated with Tofacitinib (0.5μM). 24 hours post-transfection, cells were subjected to a luciferase assay. The relative luciferase activity corresponds to the firefly luciferase light emission values divided by the renilla luciferase light emission values. Results are mean ± standard deviation of three different experiments, each performed in triplicate. Kruskal-Wallis test with Dunn Correction was performed to determine p-values (*p<0.05).

FIGURE 10. *Expression of JAK3 mutants in bone marrow cells of Balb/c mice leads to distinct leukemia phenotypes-* Spleen and bone marrow cells of diseased animals were analysed by flow cytometry with anti-CD4, anti-CD8, anti-Gr1 and anti-B220 antibodies. Cells were gated for GFP expression. FACS analysis representative of a CD8+ T-lymphoproliferation in a JAK3^{V674A} mouse **(A)**, a CD4/8+ T-lymphoproliferation in a JAK3^{L857P} mouse **(B)**, a Gr1+ myeloproliferation in a JAK3$^{L857P/Y100A}$ mouse **(C)**, a CD8+ T-lymphoproliferation in a JAK3$^{L857P/Y100A}$ mouse **(D)** and a B220+ B-lymphoproliferation in a JAK3^{L857P} mouse **(E)** are shown, either as dot plots (spleen) or as stacked column charts representing the percentage of cells positive for the different markers (bone marrow).

FIGURE 11. *Schematic representation of JAK3 mutants' activity under JAK inhibitor treatment*

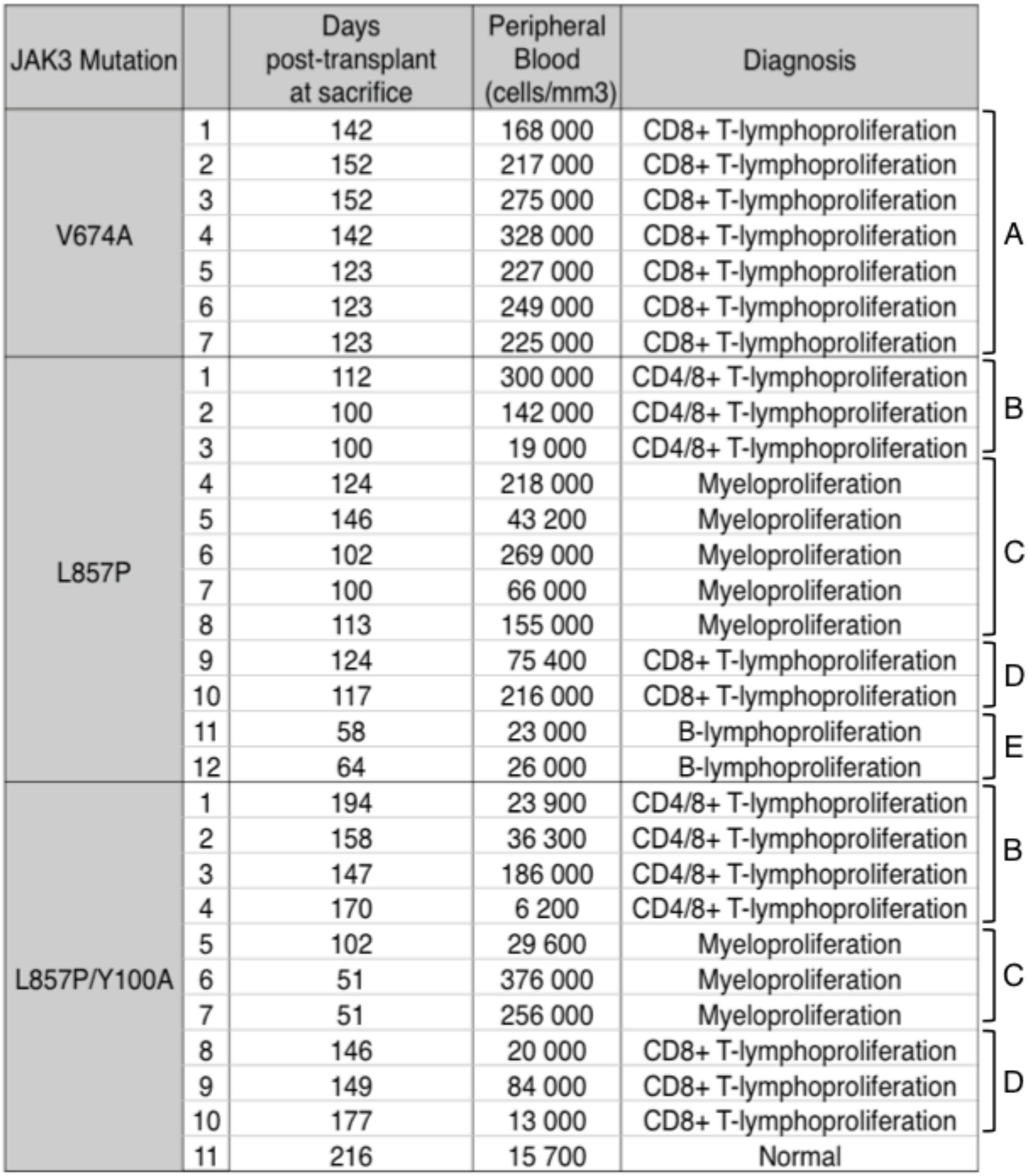
Table 1

TABLE 1. *Calculated IC50 values (µM) of NIBR3049, Ruxolitinib and Tofacitinib in autonomous Ba/F3 cells-* The table shows the calculated IC50 values (in µM) of autonomous Ba/F3 cells stably transduced with ALL-associated JAK3 mutants (V674A and L857P) or double mutant (L857P/Y100A), treated with Ruxolitinib, NIBR3049 and Tofacitinib. The IC50 mean values and standard error of the mean (SEM) values were calculated on means of triplicates cultures from three different experiments.

IC50 (µM)	JAK1/2 inhibitor		JAK3 inhibitor		JAK1/3 inhibitor	
	Ruxolitinib		NIBR3049		Tofacitinib	
	Mean	SEM	Mean	SEM	Mean	SEM
Ba/F3 JAK3^{V674A}	0.1	0.03	3.42	0.22	0.17	0.05
Ba/F3 JAK3^{L857P}	1.26	0.2	1.15	0.21	0.32	0.03
Ba/F3 JAK3$^{L857P/Y100A}$	2.33	0.21	1.31	0.24	0.44	0.05

Table 2

TABLE 2. *Expression of JAK3 mutants in bone marrow cells of Balb/c mice leads to distinct leukemia phenotypes*- The table shows the days post-transplant at sacrifice, the white blood cells (WBC) count at sacrifice, and the diagnosis of Balb/c mice receiving a bone marrow transplantation of lineage negative cells expressing JAK3^{V674A}, JAK3^{L857P} or JAK3$^{L857P/Y100A}$. Five different diagnosis were observed: CD8+ T-lymphoproliferation in JAK3^{V674A} mice **(A)**, CD4/8+ T-lymphoproliferation in JAK3^{L857P} and JAK3$^{L857P/Y100A}$ mice **(B)**, Gr1+ myeloproliferation in JAK3^{L857P} and JAK3$^{L857P/Y100A}$ mice **(C)**, another type of CD8+ T-lymphoproliferation in JAK3^{L857P} and JAK3$^{L857P/Y100A}$ mice **(D)** and B220+ B-lymphoproliferation in a JAK3^{L857P} mice **(E)**



JAK3 Mutation		Days post-transplant at sacrifice	Peripheral Blood (cells/mm3)	Diagnosis	
V674A	1	142	168 000	CD8+ T-lymphoproliferation	A
	2	152	217 000	CD8+ T-lymphoproliferation	
	3	152	275 000	CD8+ T-lymphoproliferation	
	4	142	328 000	CD8+ T-lymphoproliferation	
	5	123	227 000	CD8+ T-lymphoproliferation	
	6	123	249 000	CD8+ T-lymphoproliferation	
	7	123	225 000	CD8+ T-lymphoproliferation	
L857P	1	112	300 000	CD4/8+ T-lymphoproliferation	B
	2	100	142 000	CD4/8+ T-lymphoproliferation	
	3	100	19 000	CD4/8+ T-lymphoproliferation	
	4	124	218 000	Myeloproliferation	C
	5	146	43 200	Myeloproliferation	
	6	102	269 000	Myeloproliferation	
	7	100	66 000	Myeloproliferation	
	8	113	155 000	Myeloproliferation	
	9	124	75 400	CD8+ T-lymphoproliferation	D
	10	117	216 000	CD8+ T-lymphoproliferation	
	11	58	23 000	B-lymphoproliferation	E
	12	64	26 000	B-lymphoproliferation	
L857P/Y100A	1	194	23 900	CD4/8+ T-lymphoproliferation	B
	2	158	36 300	CD4/8+ T-lymphoproliferation	
	3	147	186 000	CD4/8+ T-lymphoproliferation	
	4	170	6 200	CD4/8+ T-lymphoproliferation	
	5	102	29 600	Myeloproliferation	C
	6	51	376 000	Myeloproliferation	
	7	51	256 000	Myeloproliferation	
	8	146	20 000	CD8+ T-lymphoproliferation	D
	9	149	84 000	CD8+ T-lymphoproliferation	
	10	177	13 000	CD8+ T-lymphoproliferation	
	11	216	15 700	Normal	

Figure 1

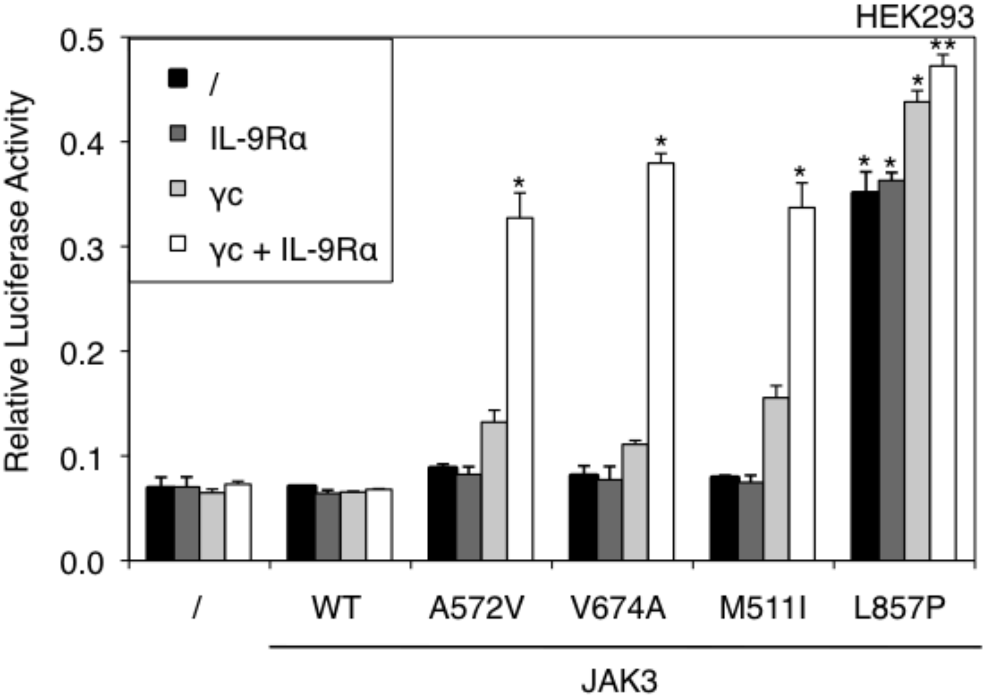

HEK293
Relative Luciferase Activity
0.5
0.4
0.3
0.2
0.1
0.0
■ /
■ IL-9Rα
■ γc
□ γc + IL-9Rα
*
**
/
WT
A572V
V674A
M511I
L857P
JAK3

Figure 2

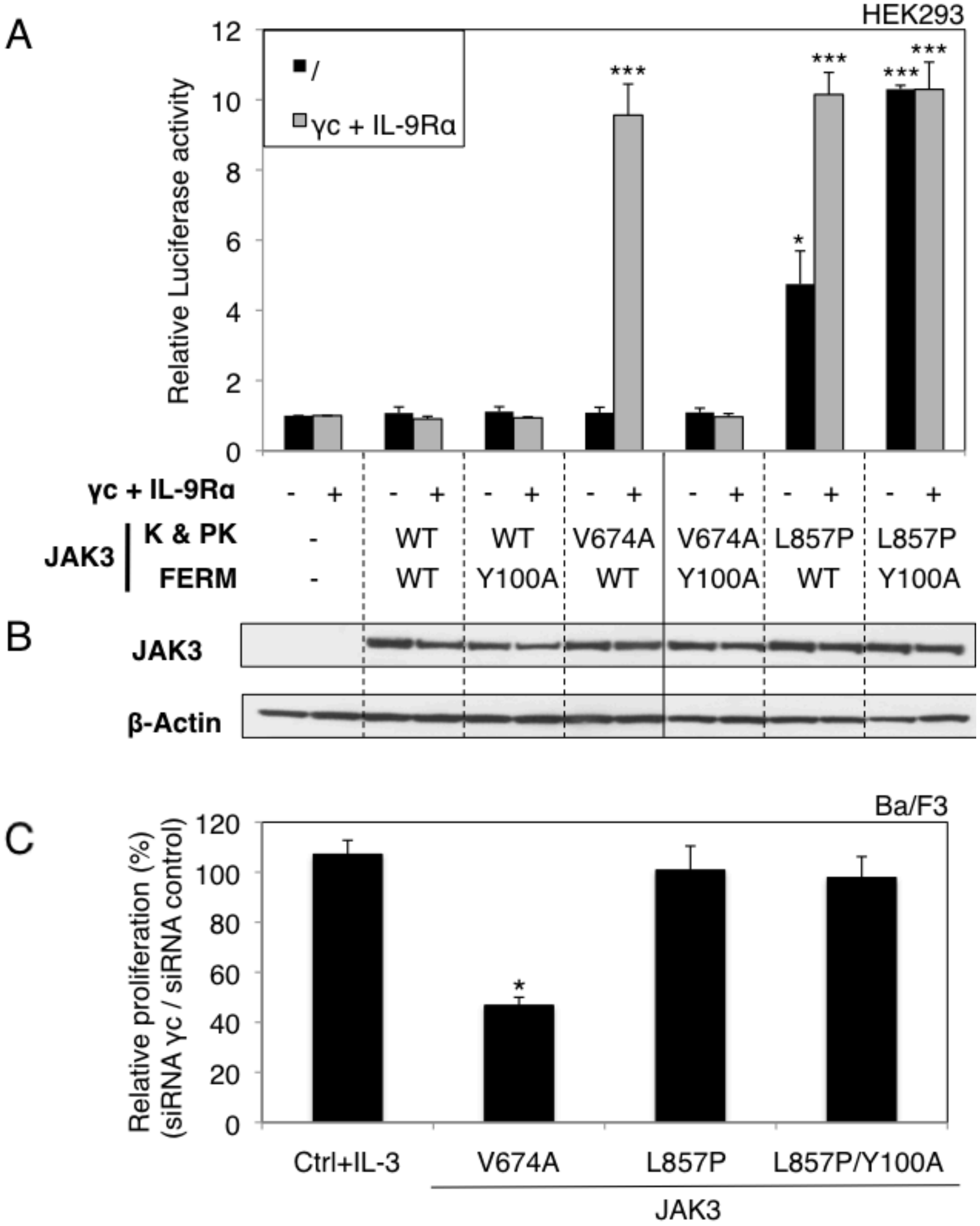

A
HEK293
Relative Luciferase activity
0
2
4
6
8
10
12
■ /
▨ γc + IL-9Rα
***
*
***
***
***
γc + IL-9Rα
- + - + - + - + - + - + - +
JAK3
K & PK
FERM
-
-
WT
WT
WT
Y100A
V674A
WT
V674A
Y100A
L857P
WT
L857P
Y100A
B
JAK3
β-Actin
C
Ba/F3
Relative proliferation (%)
(siRNA γc / siRNA control)
0
20
40
60
80
100
120
*
Ctrl+IL-3
V674A
L857P
L857P/Y100A
JAK3

Figure 3

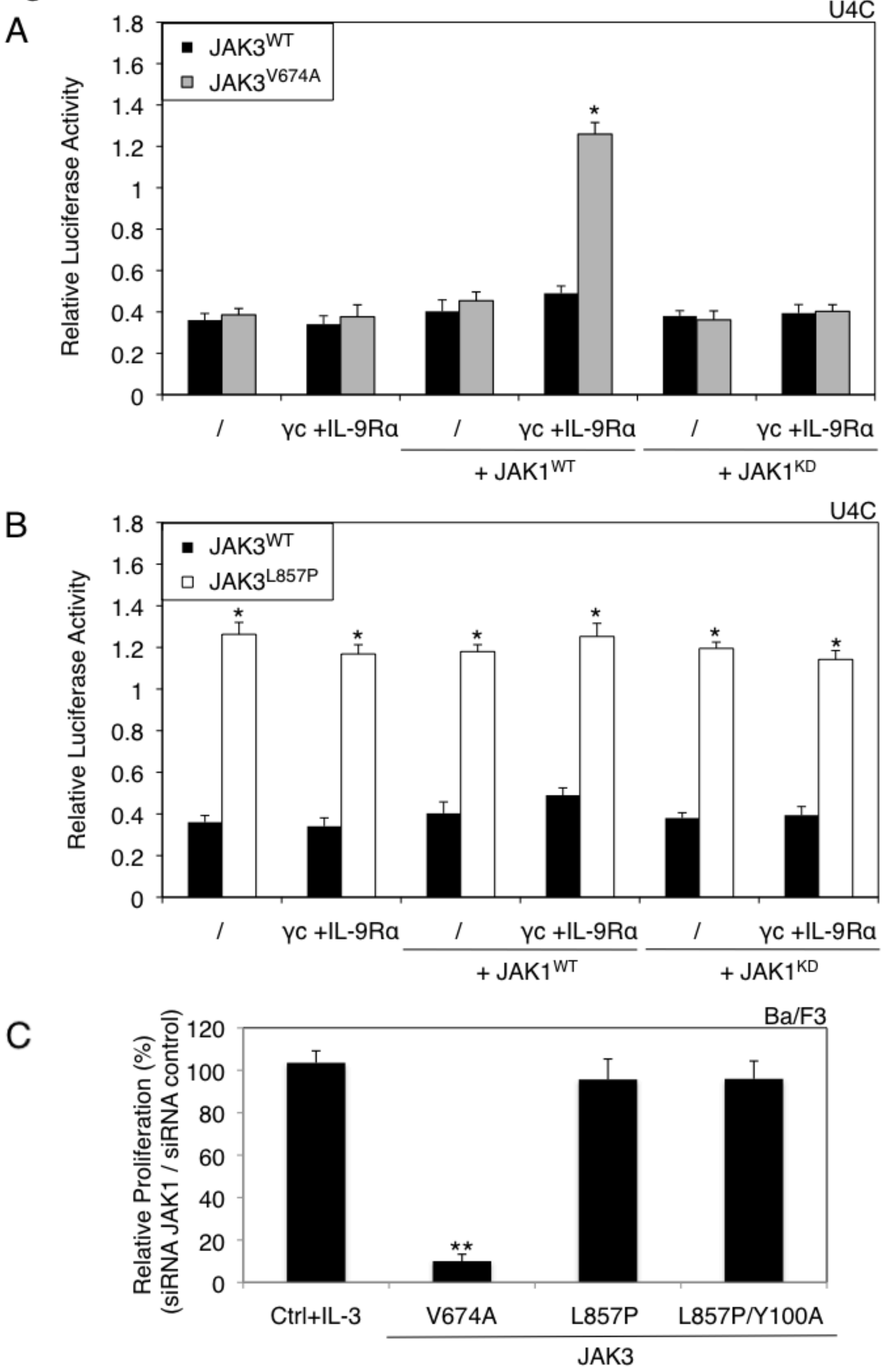

A
U4C
Relative Luciferase Activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
■ JAK3^{WT}
▨ JAK3^{V674A}
*
/
γc +IL-9Rα
/
γc +IL-9Rα
/
γc +IL-9Rα
+ JAK1^{WT}
+ JAK1^{KD}
B
U4C
Relative Luciferase Activity
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
■ JAK3^{WT}
☐ JAK3^{L857P}
*
*
*
*
*
*
/
γc +IL-9Rα
/
γc +IL-9Rα
/
γc +IL-9Rα
+ JAK1^{WT}
+ JAK1^{KD}
C
Ba/F3
Relative Proliferation (%) (siRNA JAK1 / siRNA control)
120
100
80
60
40
20
0
**
Ctrl+IL-3
V674A
L857P
L857P/Y100A
JAK3

Figure 4

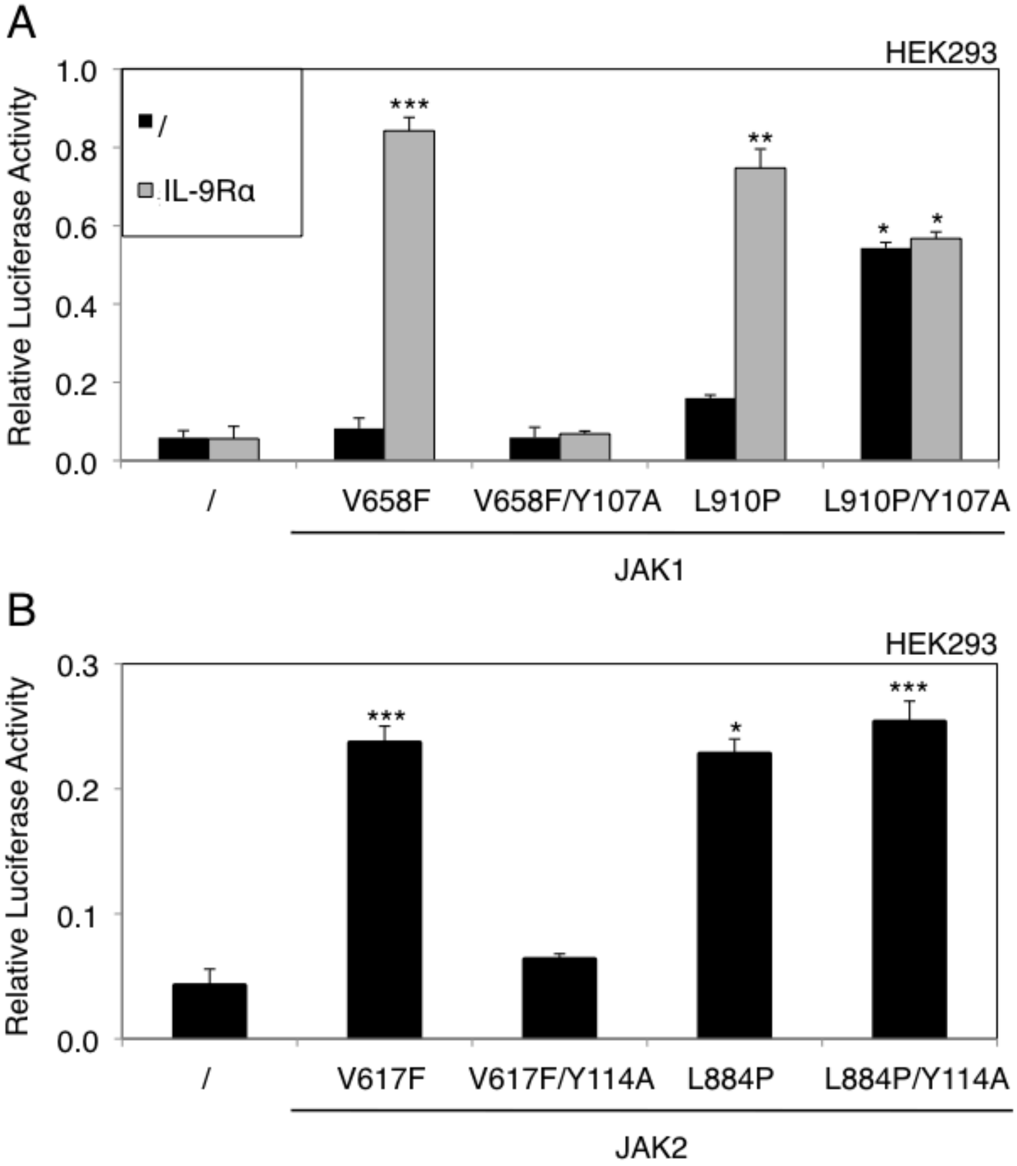

A
HEK293
Relative Luciferase Activity
1.0
0.8
0.6
0.4
0.2
0.0
■ /
▨ IL-9Rα
***
**
*
*
/
V658F
V658F/Y107A
L910P
L910P/Y107A
JAK1
B
HEK293
Relative Luciferase Activity
0.3
0.2
0.1
0.0
***
*
***
/
V617F
V617F/Y114A
L884P
L884P/Y114A
JAK2

Figure 5

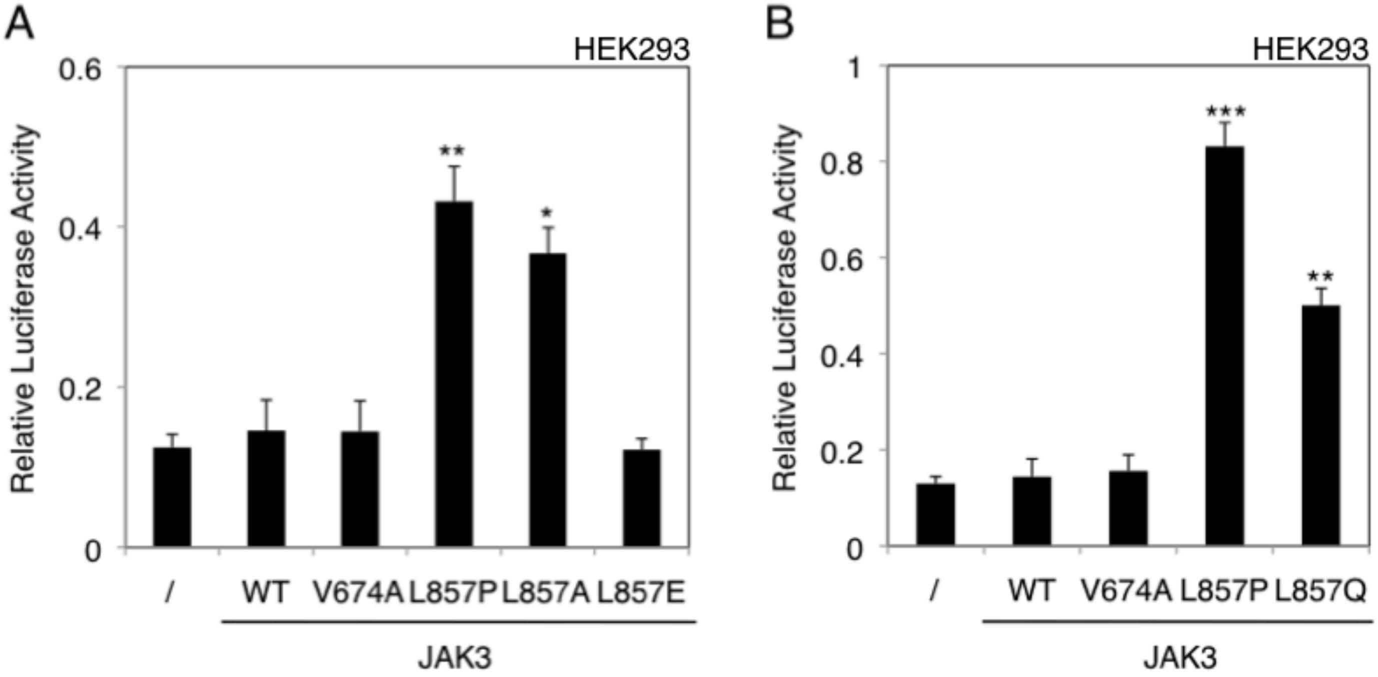

A
HEK293
Relative Luciferase Activity
0.6
0.4
0.2
0
**
*
/
WT
V674A
L857P
L857A
L857E
JAK3
B
HEK293
Relative Luciferase Activity
1
0.8
0.6
0.4
0.2
0
***
**
/
WT
V674A
L857P
L857Q
JAK3

Figure 6

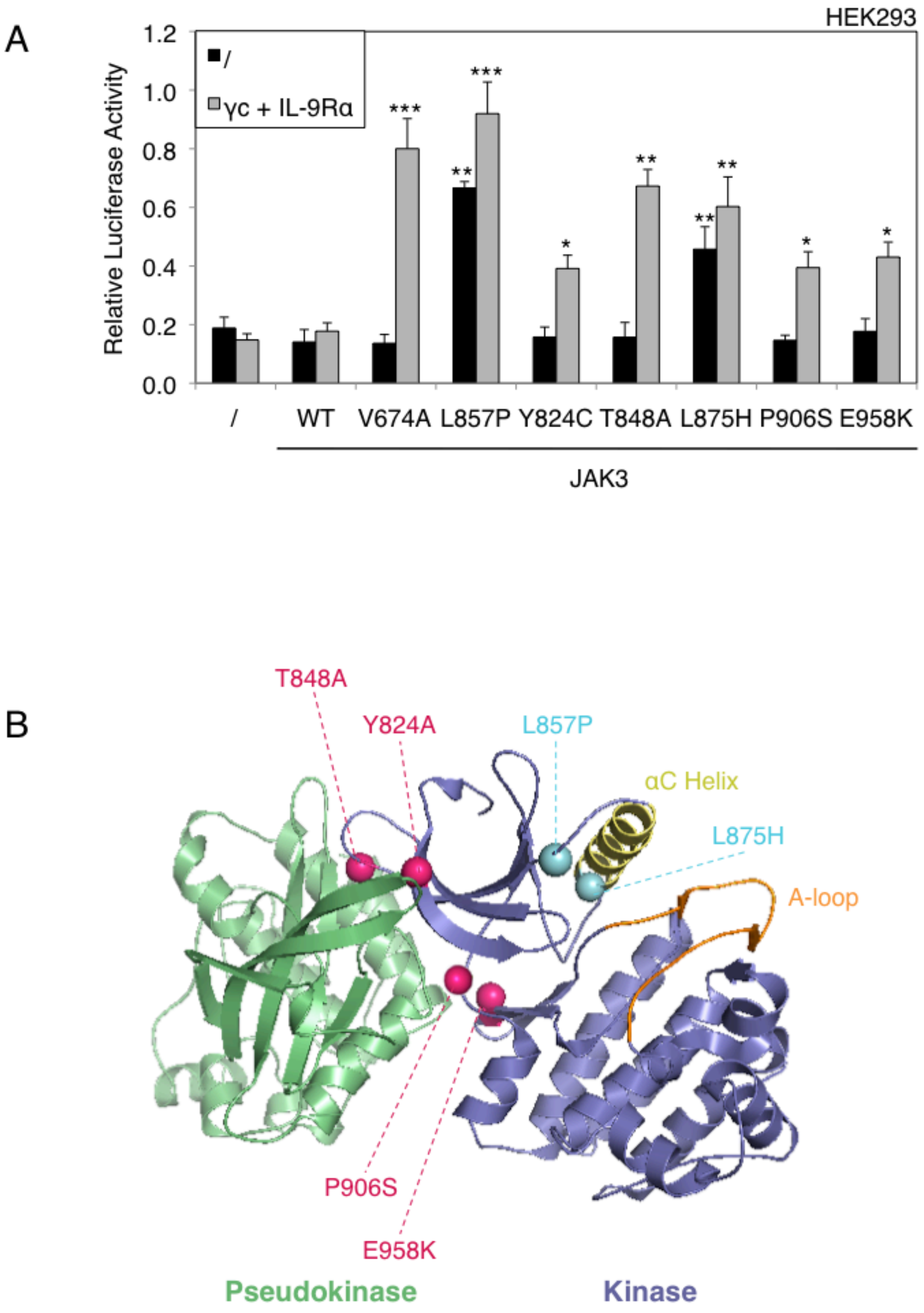

A
HEK293
■ /
▨ γc + IL-9Rα
Relative Luciferase Activity
0.0
0.2
0.4
0.6
0.8
1.0
1.2
***
***
**
*
**
**
**
*
*
/
WT
V674A
L857P
Y824C
T848A
L875H
P906S
E958K
JAK3
B
T848A
Y824A
L857P
αC Helix
L875H
A-loop
P906S
E958K
Pseudokinase
Kinase

Figure 7

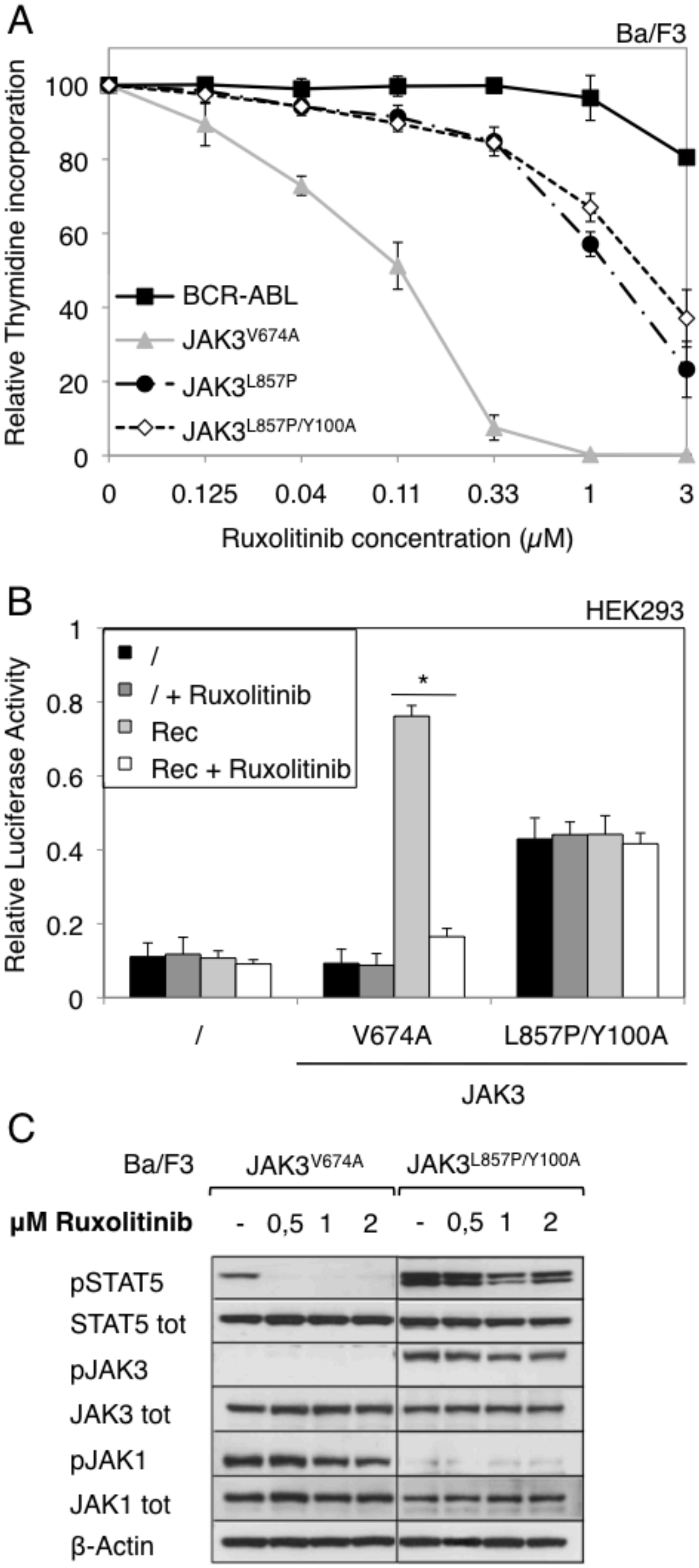

A
Ba/F3
Relative Thymidine incorporation
100
80
60
40
20
0
BCR-ABL
JAK3V674A
JAK3L857P
JAK3L857P/Y100A
0
0.125
0.04
0.11
0.33
1
3
Ruxolitinib concentration (μM)
B
HEK293
Relative Luciferase Activity
1
0.8
0.6
0.4
0.2
0
■ /
■ / + Ruxolitinib
■ Rec
□ Rec + Ruxolitinib
*
/
V674A
L857P/Y100A
JAK3
C
Ba/F3
JAK3V674A
JAK3L857P/Y100A
μM Ruxolitinib
- 0,5 1 2
- 0,5 1 2
pSTAT5
STAT5 tot
pJAK3
JAK3 tot
pJAK1
JAK1 tot
β-Actin

Figure 8

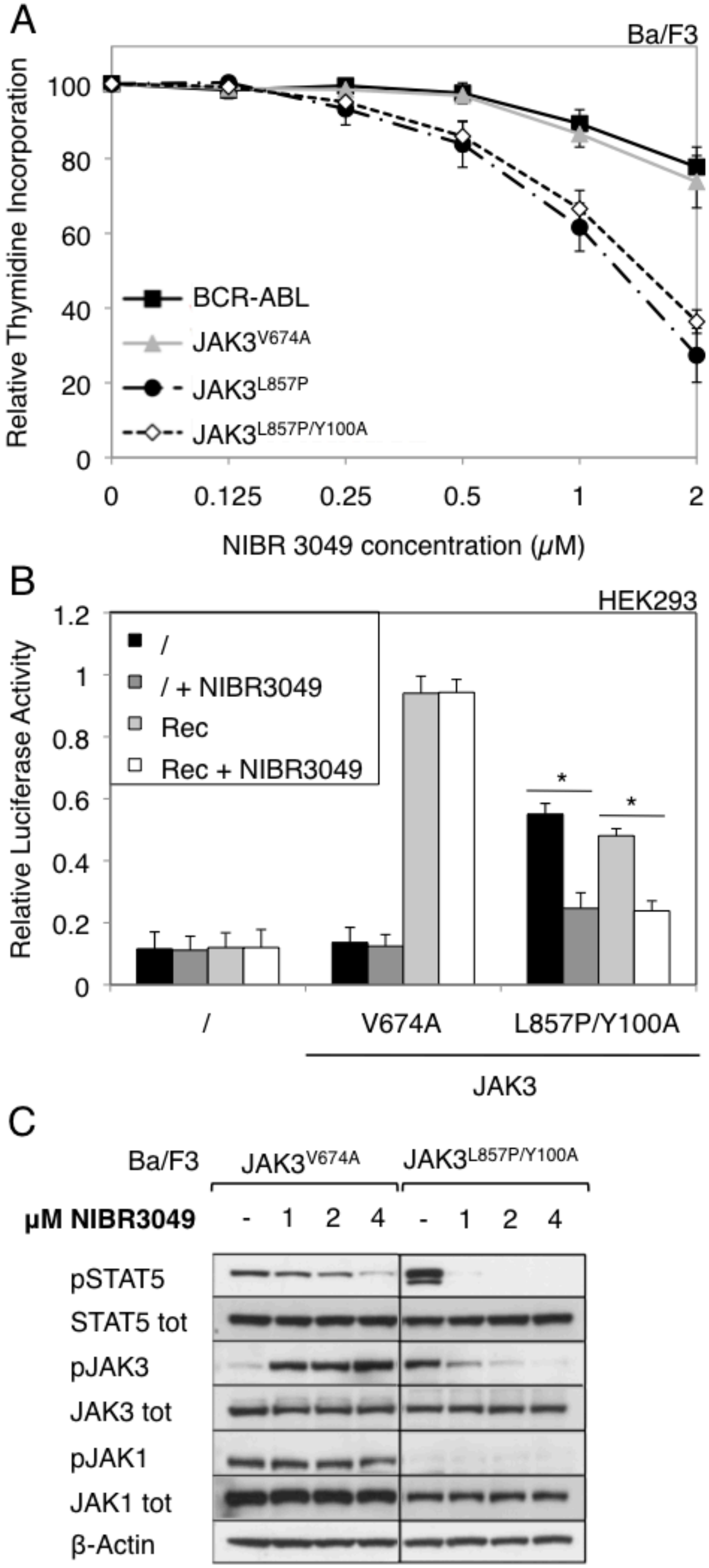

A
Ba/F3
Relative Thymidine Incorporation
100
80
60
40
20
0
0
0.125
0.25
0.5
1
2
NIBR 3049 concentration (μM)
BCR-ABL
JAK3V674A
JAK3L857P
JAK3L857P/Y100A
B
HEK293
Relative Luciferase Activity
1.2
1
0.8
0.6
0.4
0.2
0
/
/ + NIBR3049
Rec
Rec + NIBR3049
*
*
/
V674A
L857P/Y100A
JAK3
C
Ba/F3
JAK3V674A
JAK3L857P/Y100A
μM NIBR3049
- 1 2 4
- 1 2 4
pSTAT5
STAT5 tot
pJAK3
JAK3 tot
pJAK1
JAK1 tot
β-Actin

Figure 9

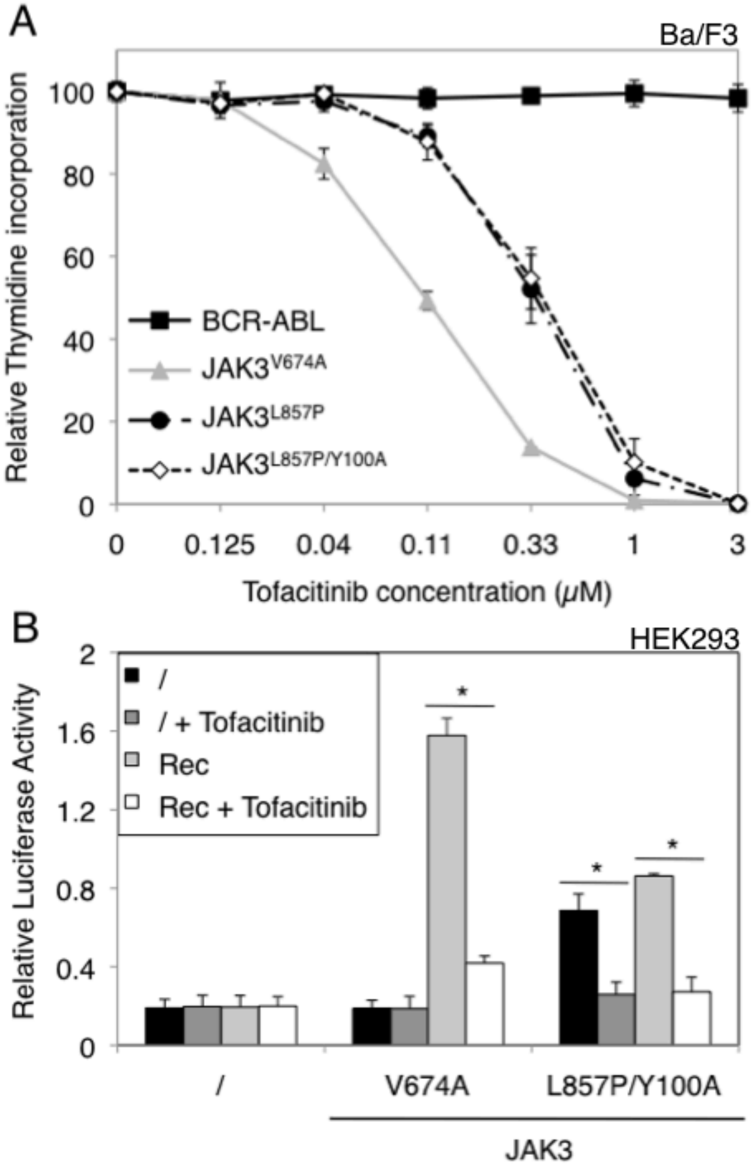

A
Ba/F3
Relative Thymidine incorporation
100
80
60
40
20
0
BCR-ABL
JAK3^{V674A}
JAK3^{L857P}
JAK3$^{L857P/Y100A}$
0
0.125
0.04
0.11
0.33
1
3
Tofacitinib concentration (μM)
B
HEK293
Relative Luciferase Activity
2
1.6
1.2
0.8
0.4
0
/
/ + Tofacitinib
Rec
Rec + Tofacitinib
*
*
*
/
V674A
L857P/Y100A
JAK3

Figure 10

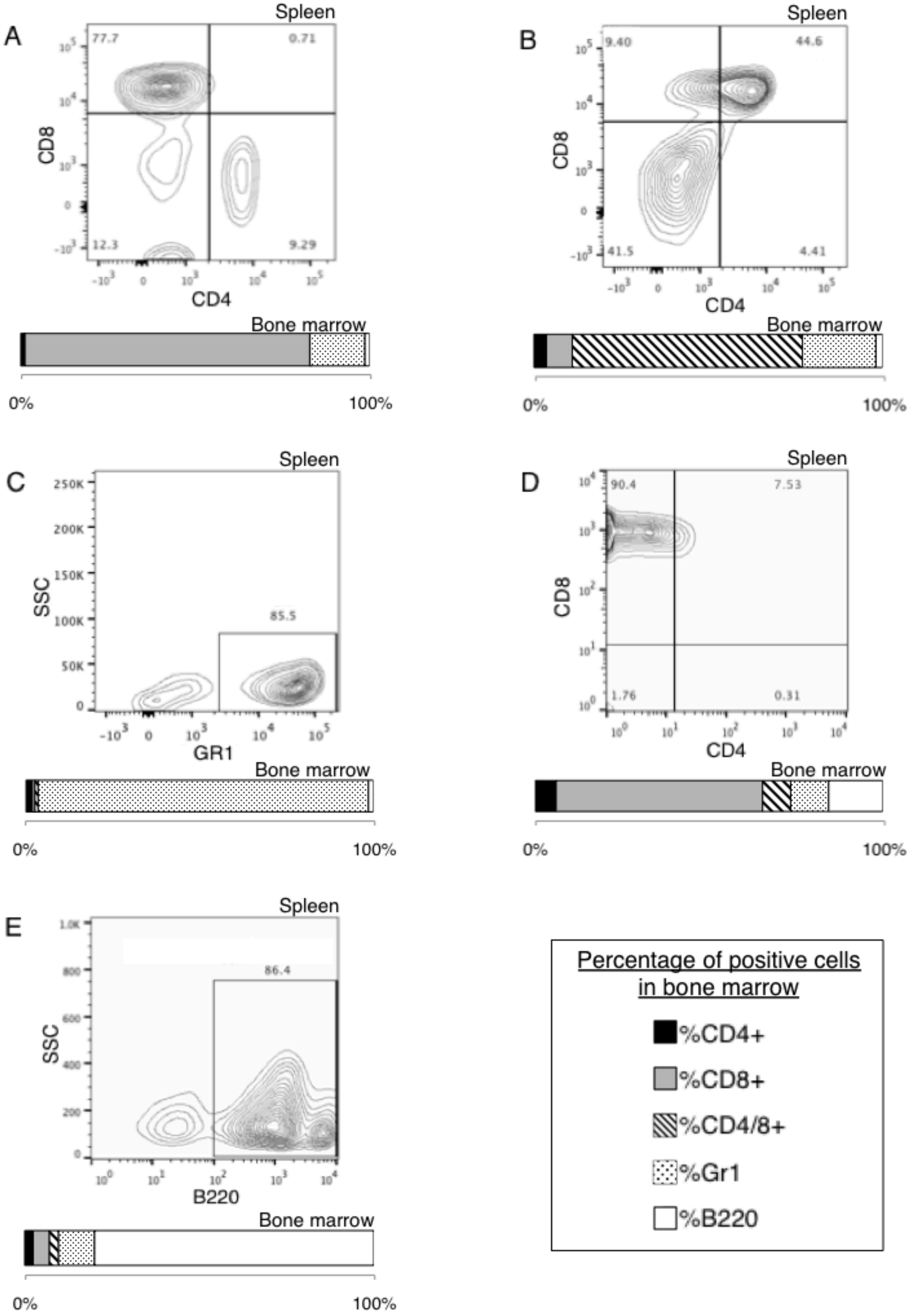
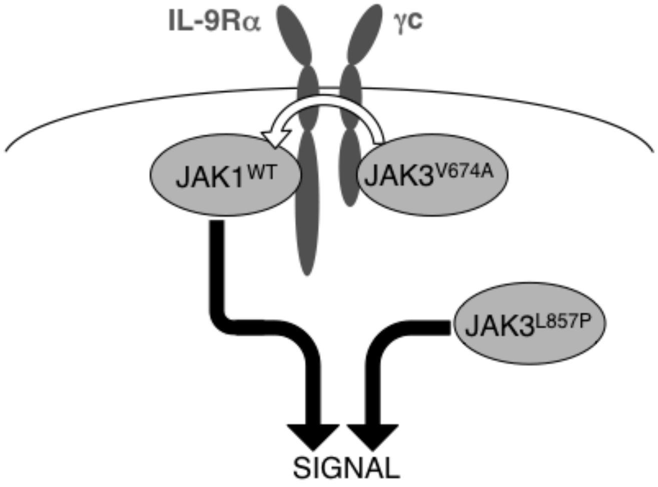
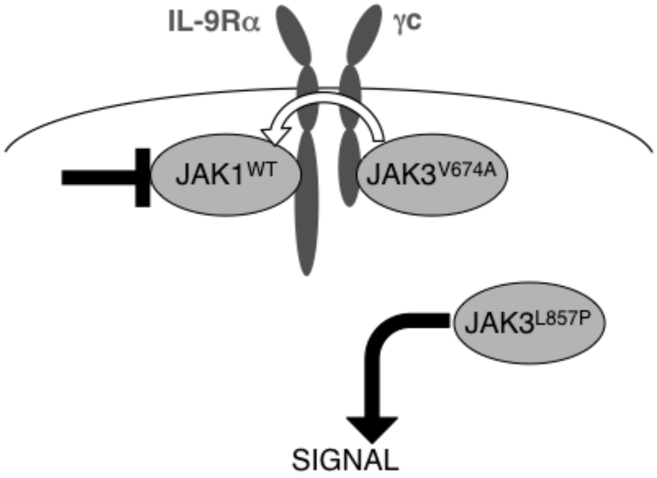

A
Spleen
77.7
0.71
12.3
9.29
CD8
CD4
Bone marrow
0%
100%
B
Spleen
9.40
44.6
41.5
4.41
CD8
CD4
Bone marrow
0%
100%
C
Spleen
85.5
SSC
GR1
250K
200K
150K
100K
50K
Bone marrow
0%
100%
D
Spleen
90.4
7.53
1.76
0.31
CD8
CD4
Bone marrow
0%
100%
E
Spleen
86.4
SSC
B220
Bone marrow
0%
100%
Percentage of positive cells in bone marrow
■%CD4+
▨%CD8+
▧%CD4/8+
⁙%Gr1
☐%B220

Figure 11

A. No Inhibitor



B. Ruxolitinib



C. NIBR3049