CHAPTER III

Results

Murine Lymphoid Cell Lines Acquire Cytokine Independent Cell Growth Through Spontaneous JAK1 Activating Mutations Scattered Throughout the Kinase and Pseudokinase Domains

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Preamble

As mentioned above, the primary aim of my thesis was to use the BaF3 phe116 *in vitro* model to better understand the mechanisms involved in multiple-step tumorigenesis processes associated with constitutive STAT5 activation.

The first approach I used was based on microarray analysis, looking for up- or down-regulation of gene expression, or for putative transcriptional signatures revealed by clustering of autonomous clones. This work turned out mainly unsuccessful, except for the identification of a minority (<10%) of clones that overexpressed IL-3, and therefore activated an autocrine loop. Other microarray experiments focusing on microRNA regulation were even more deceptive and did not lead to any exploitable result. Our last attempt consisted in sequencing JAK1 in autonomous clones, and this led to the observations reported in the manuscript that follows.

In this paper, we show that 80% of the BaF3 autonomous clones selected from the cells that overcame impaired IL-9 responsiveness by overexpressing spontaneously JAK1, acquired single point mutation in the kinase or pseudokinase domain of JAK1. Retroviral transduction of these JAK1 mutants in BaF3 cells results in cytokine independent proliferation supporting our conclusion that JAK1 mutations represent the second genetic hit associated with constitutive activation of JAK-STAT pathway in our model.

Murine lymphoid cell lines acquire cytokine independent cell growth through spontaneous JAK1 activating mutations scattered throughout the kinase and pseudokinase domains

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ABSTRACT

The acquisition of growth signal self-sufficiency is one of the hallmarks of cancer cells. We previously reported that the murine cytokine-dependent BaF3 cell line can become autonomous and tumorigenic through a two step selection process *in vitro*. Selection of cells able to proliferate in response to a defective IL-9 receptor was associated with JAK1 overexpression, and rendered these cells prone to a second selection step towards autonomously growing tumorigenic cells. Here, we show that 80% of the autonomous clones resulting from this process harbor heterozygous mutations in the kinase or pseudokinase domains of JAK1. We characterized 18 *de novo* JAK1 mutations that were able to constitutively activate the JAK/STAT pathway when co-expressed with JAK1-binding cytokine receptors in HEK293 cells. Four of the 18 spontaneous mutations were similar to mutations already described in human tumors. Retroviral transduction of JAK1 mutants in the parental BaF3 cell line recapitulated the phenotype of the autonomous clones, confirming the gain of function property of these mutations. 13 out of the 18 mutants conferred hypersensitivity to the anti-proliferative effect of type I IFN. Most JAK1 mutants also remained sensitive to an ATP-competitive JAK inhibitor, with the exception of the F958V, F958C and K1026E mutations that rendered JAK1 not only constitutively active, but also resistant to the JAK inhibitor. Taken together, these data show that cytokine-dependent hematopoietic progenitors acquire tumorigenic properties through a two-step process consisting of overexpression and activating mutations in JAK1.

INTRODUCTION

Aberrant activation of cytokine and growth factor signaling pathways are well known characteristics of all types of leukemia and tumors. In normal cells, cytokines provide the stimulus for proliferation, survival, self-renewal, differentiation and functional activation. In leukemic cells, like in other cancers, these pathways are usurped to subserve critical aberrant properties of the malignant transformation (1). Beside the ability to overcome negative growth signals and apoptosis, and to spread to different tissues and organs, the self-sufficiency in growth signals is one of the characteristics of malignant cell. In the process of tumorigenesis, cytokines are known to play an important role as autocrine or paracrine growth-promoting factors (2). However, the efficacy of most anti-tumor treatments targeting cytokines turned out to be elusive, partly because tumor cells tend to acquire cytokine-independent growth properties. One of the best examples is the role of IL-6 in multiple myeloma development. In early disease, paracrine secretion of IL-6 by bone marrow stromal

cells, is considered to be a key growth-promoting and anti-apoptotic factor for the transformed plasma cell. With time, myeloma cells acquire other genetic abnormalities, such as activating mutations in N-RAS and K-RAS, which bypass the need of IL-6 to activate the MAPKinase pathway, thus allowing IL-6-independent survival, proliferation and further tumor progression (3). These and other acquired genetic abnormalities in genes involved in cytokine signal transduction might explain why anti-IL-6 or/and anti-IL-6R therapy exhibited only moderate and transient beneficial effects in myeloma patients (3,4).

To analyze the mechanisms responsible for the spontaneous transformation of cytokine-dependent cell lines towards growth factor-independent tumorigenic clones, we developed a simplified *in vitro* model of tumorigenesis that implies two genetic events. This model stemmed from the study of IL-9 receptor signal transduction in IL-3-dependent BaF3 cells, which normally die after cytokine withdrawal. Beside the role of IL-9 in TH2 immune responses, numerous observations suggest that dysregulated IL-9 expression and/or signal transduction is implicated in T cell tumorigenesis (5). IL-9 mediates its activities via a heterodimeric receptor complex formed by the IL-9Rα chain (IL-9Rα), which associates with JAK1, and the IL-2Rγ chain, also called γc (common γ chain), which associates with JAK3. Upon IL-9 binding, JAK1 and JAK3 are cross-activated and IL-9Rα is phosphorylated. A single phospho-tyrosine (Y116) is responsible for the recruitment and activation of STATs by IL-9Rα (6). BaF3 cells transfected with an IL-9Rα mutant lacking this STAT-recruiting site (BaF3 phe116) almost completely failed to proliferate and activate STATs in response to IL-9. However, in a prolonged culture with IL-9, a small number of cells could manage to survive and even proliferate, allowing the selection of an IL-9-dependent cell line (BaF3 phe116/9) (7). In contrast to parental BaF3 phe116 cells, those IL-9-selected cells could progress to autonomous cells (BaF3 A) after a second step of selection without cytokine. These autonomous cells show a cytokine-independent activation of JAK1 and STAT5 and are highly tumorigenic when injected in mice, which was not the case for BaF3 phe116 and BaF3 phe116/9 (7,8).

We previously showed that JAK1 overexpression was associated to the first step of transformation, namely selection for IL-9-responsive BaF3 phe116/9 cells (8). We also showed that JAK1 ectopic overexpression increased sensitivity of BaF3 phe116 cells to IL-9 and promoted progression to cytokine-independent BaF3 autonomous cells. JAK1 is a member of the Janus kinase family comprising three other mammalian non-receptor tyrosine kinases (JAK2, JAK3 and TYK2) that associate to cytokine receptors without intrinsic kinase

activity to mediate cytokine-induced signal transduction (9). Like other JAKs, JAK1 contains a tyrosine kinase domain at the C-terminus, which is flanked by a catalytically inactive pseudokinase domain with regulatory activity. The N-teminal segment consists of a FERM domain that mediates association to membrane-proximal region of the cytokine receptors (10) and an atypical SH2 domain of undetermined function (11).

In this study, we show that 80% of the BaF3 autonomous clones selected from the cells that overcame impaired IL-9 responsiveness by overexpressing JAK1, acquired a single activating point mutation in the kinase or pseudokinase domain of JAK1. These JAK1 mutations represent a second genetic event providing tumorigenic potential to the cells by inducing constitutive activation of JAK-STAT pathway that support their autonomous proliferation. Recently, we and others reported that JAK1 mutations occur in acute lymphoblastic leukemia (ALL) and are relatively common in adult patients with T cell ALL (12-14). By generating a collection of autonomous clones with a broad spectrum of spontaneous activating mutations, the *in vitro* model described here provide us with a unique tool to study the biological and functional characteristics of tumor cells harboring JAK1 mutations in one of their autologous allele. This will be illustrated here with the sensitivity to the anti-proliferative effect of type 1 IFN and of ATP-competitive JAK1 inhibitors.

RESULTS

JAK1 mutations occur frequently during the selection of BaF3 autonomous clones. BaF3 cells transfected with a mutated hIL-9R (BaF3 phe116), defective in STAT recruitment and activation, poorly respond to IL-9. However, after selection with this factor, we obtained cell lines, designated BaF3 phe116/9, which proliferated well in IL-9. After cytokine withdrawal, those cells, in contrast to the parental cells, gave rise to autonomous BaF3 clones, which were highly tumorigenic *in vivo* (7,8). We showed previously that JAK1 overexpression was the key genetic event involved in the first step of transformation. Overexpression of JAK1 not only increased the sensitivity to IL-9 but also allowed a second selection step toward cytokine-independent growth with constitutive STAT activation. To characterize the genetic events associated with the second selection step, we compared by microarray the genes expressed in the cytokine-dependent BaF3 phe116/9 cell line and in different autonomous clones. We found very similar gene expression profiles (data not shown), suggesting that growth of autonomous clones was also induced by the activation of the JAK-STAT pathway. As constitutive phosphorylation of JAK1 was detectable in at least some of these autonomous clones, we sequenced JAK1 cDNA from 110 independent autonomous clones and found that

88 of them were heterozygous for a single *de novo* mutation. Altogether, 18 different missense mutations were identified as illustrated in Figure 1 and Table 1.

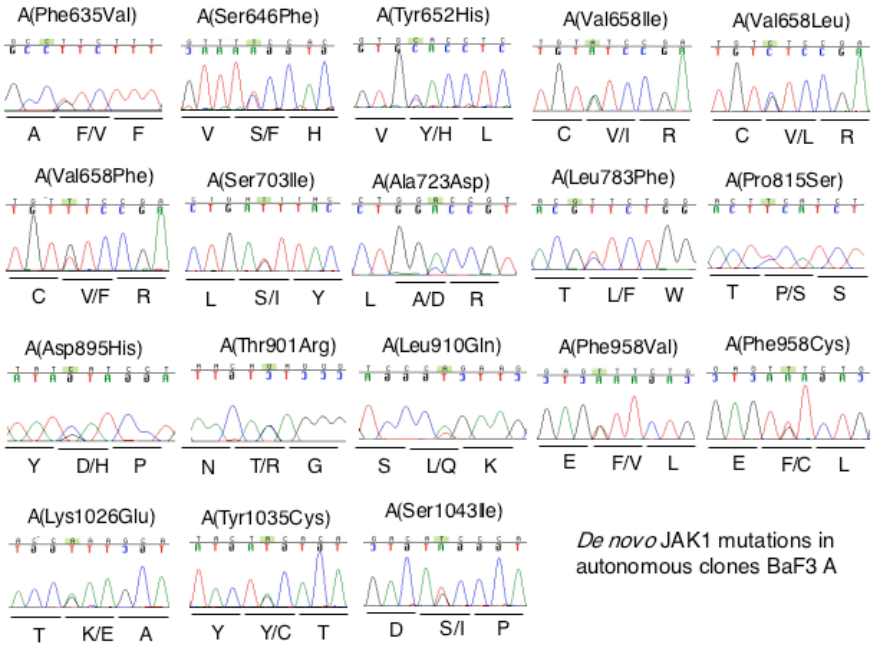

FIGURE 1 De novo JAK1 mutations occur frequently during the selection of BaF3 autonomous clones. *A) Autonomously (BaF3 A) clones were selected from IL-9 responding (BaF3 phe116/9) cells. Direct sequencing of JAK1 RT-PCR product from BaF3 A clones, showing de novo point mutations in JAK1 in autonomous cells. The JAK1 sequence of one representative autonomous clone is shown for each of the 18 JAK1 mutations from total of 88 clones heterozygous for a single JAK1 mutation.*

JAK1 MUTATION	NUCLEOTIDE CHANGE	LOCALIZATION IN STRUCTURE	FREQUENCY OF MUTATION
F635V	**T**TC>**G**TC	pseudokinase	9/88
S646F	T**C**C>T**T**C	pseudokinase	1/88
Y652H	**T**AC>**C**AC	pseudokinase	6/88
V658I	**G**TC>**A**TC	pseudokinase	2/88
V658L	**G**TC>**C**TC	pseudokinase	4/88
V658F	**G**TC>**T**TC	pseudokinase	2/88
S703I	A**G**T>A**T**T	pseudokinase	13/88
A723D	G**C**C>G**A**C	pseudokinase	1/88
L783F	**C**TC>**T**TC	pseudokinase	8/88
P815S	**C**CA>**T**CA	pseudokinase	1/88
D895H	**G**AT>**C**AT	kinase	1/88
T901R	A**C**A>A**G**A	kinase	1/88
L910Q	CTG>C**A**G	kinase	1/88
F958V	**T**TT>**G**TT	kinase	19/88
F958C	T**T**T>T**G**T	kinase	11/88
K1026E	**A**AA>**G**AA	kinase	2/88
Y1035C	T**A**C>T**G**C	kinase	4/88
S1043I	A**G**C>A**T**C	kinase	2/88

TABLE 1 De novo mutations in JAK1 identified in vitro in autonomous clones. *List of mutated residues in JAK1 with their nucleotide and amino acid change, localization within the JAK1 structure and frequency of mutation.*

JAK1-mutated BaF3 autonomous cells are characterized by the constitutive activation of JAK1, STAT5 and the MAPKinase pathway. We previously showed that progression of BaF3 phe116/9 cells toward cytokine-independence was associated with a strong constitutive activation of STAT5 (7). We therefore examined whether all JAK1-mutated autonomous clones showed similar signaling profile. We chose one representative JAK1 mutation-positive autonomous BaF3 clone for each of the 18 JAK1 mutations and used Western Blot to assess the activation status of JAK1, STAT5 and ERK1/2.

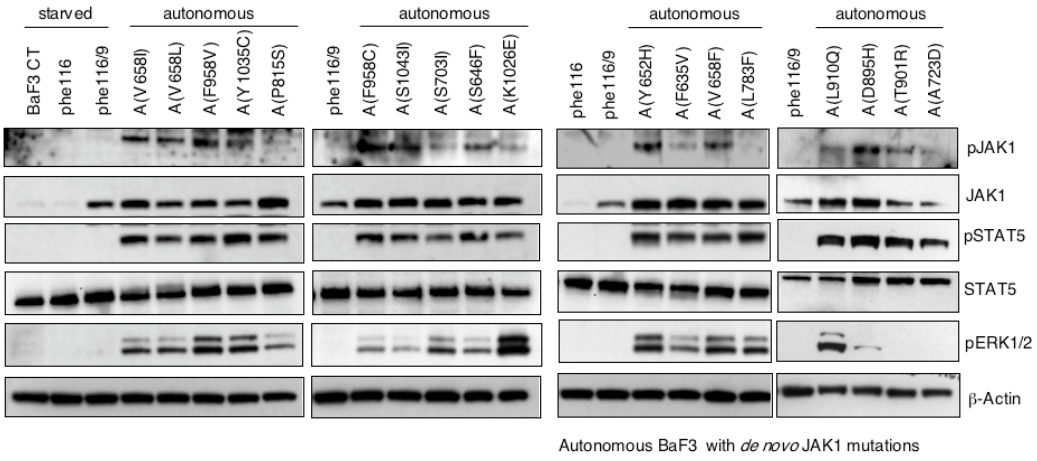

FIGURE 2 JAK1 mutated BaF3 autonomous cells are characterized by constitutive activation of JAK1, STAT5 and the MAPKinase pathway. *10^6 BaF3 phe116, BaF3 phe116/9 and autonomous BaF3 A cells were starved for 4 hours, lyzed and subjected to Western Blot analysis. Basal phosphorylation of JAK1, STAT5 and ERK1/2 was detected using specific anti-pY1034/35 JAK1, anti-pY694 STAT5, anti-pP42/44 antibodies. Membranes were reprobed with anti-JAK1 and anti-β-actin antibodies as a control. Similar results were obtained in 3 independent experiments.*

As shown in Figure 2, none of these proteins were phosphorylated in BaF3 phe116 or IL-9-selected BaF3 phe116/9 in the absence of cytokine. By contrast, all autonomous BaF3 clones showed constitutive activation of STAT5, and for most of them constitutive phosphorylation of JAK1 and MAPKinase was detected, confirming that all JAK1 mutated autonomous clones harbored constitutive activation of the JAK-STAT and the MAPkinase pathways. The total level of JAK1 was strongly increased in BaF3 phe116/9 and in autonomous clones, confirming that selection for IL-9 responsiveness was associated with spontaneous JAK1 overexpression (8).

Ectopic expression of each JAK1 mutant in BaF3 cells confers cytokine-independent cell proliferation. To confirm that the acquisition of mutations in JAK1 is responsible for autonomous BaF3 cell proliferation, we produced mutated JAK1 constructs for the first 13 of the 18 JAK1 mutations that we found. We transduced parental BaF3 cells with a bicistronic vector expressing either mutated or wild-type (WT) JAK1 together with the Green

Fluorescent Protein (GFP). After flow cytometry sorting based on GFP expression, transduced BaF3 cells were cultured with or without IL-3. In contrast to parental cells or cells stably transduced with WT JAK1, all JAK1 mutated construct were able to induce the autonomous growth of BaF3, confirming that the mutations occurring spontaneously *in vitro* are activating mutations (Figure 3A).

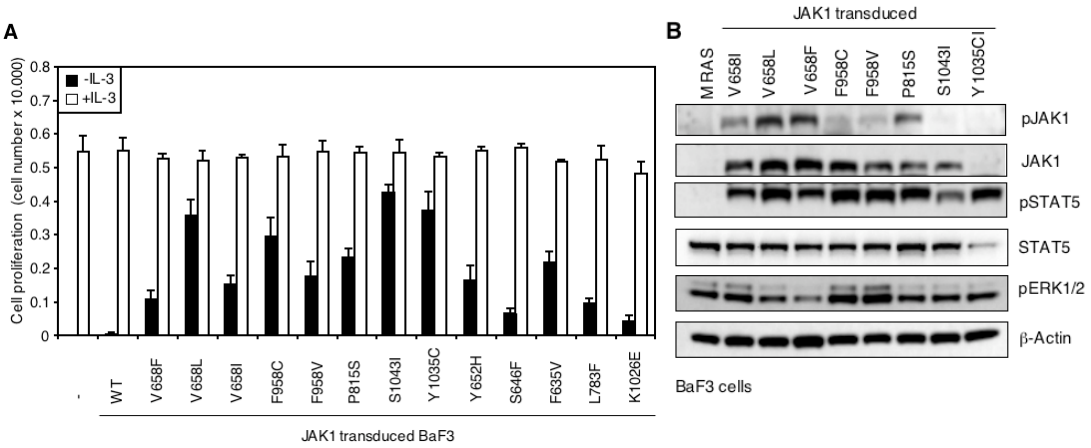

FIGURE 3 Ectopic expression of each JAK1 mutant in BaF3 cells confers cytokine-independent cell proliferation and STAT5 phosphorylation. *(A) 10^6 BaF3 cells stably transduced with JAK1 WT or different JAK1 mutants were washed three-times in PBS and seeded in 24-well plates at a concentration of 10,000 cells/ml with and without mIL-3. After 72 hours, living cells were counted in both conditions, with or without mIL-3. Results are mean ± SD of cell culture triplicates. Similar results were obtained in 3 independent experiments. (B) 10^6 autonomously proliferating BaF3 cells stably transduced with MRAS or different JAK1 mutants were lyzed and subjected to Western Blot analysis. Basal phosphorylation of JAK1, STAT5 and ERK1/2 was detected using specific anti-pY1034/35 JAK1, anti-pY694 STAT5, anti-ERK1/2antibodies. Membranes were reprobed with anti-JAK1 and anti-β-actin antibodies as a control. Similar results were obtained in 3 independent experiments.*

Consistent with these results, most of JAK1 mutants induced the constitutive phosphorylation of JAK1. All JAK1 mutants induced the constitutive activation of STAT5 and MAP kinases, when ectopically expressed in BaF3 cells as illustrated in Figure 3B for 8 mutants, similarly to autonomous BaF3 harboring spontaneously acquired mutations. As a control for this experiment, we used an autonomous BaF3 cell line obtained by transfection with an oncogenic mutant of MRAS (Glu71Lys), which shows constitutive activation of MAPK but not JAK/STAT pathway (Figure 3B).

Spontaneous activating mutations define various potential hotspots in JAK1 structure. Among the 18 different mutations identified, 10 affect residues located in the pseudokinase domain and 8 in the kinase domain (Figure 4A). Interestingly, all the nucleotides mutated in the murine JAK1 sequence were conserved in the human JAK1 sequence (data not shown). To better highlight the localization of mutated residues, we further used the three-dimensional modeled structure of kinase (JH1) and pseudokinase (JH2) domain of JAK1 previously described (12). As shown in Figure 4, half of the affected residues (6 of 10 *de novo*

mutations) of the pseudokinase domain (F635V, S646F, Y652H and V658I/L/F) are located within the pocket formed between the JH2 αC helix and an adjacent loop comprising Val658 and Tyr652 residues. These two regions of the JH2 domain were predicted to hold the activation loop of the kinase domain in the active conformation (15). The V658F mutation is homologous to the frequent JAK2 V617F mutant found in majority of the patients with MPNs (16,17). This area therefore represents a hotspot for mutations that might use a common mechanism to activate JAK1. Furthermore, this interface between the JH2 and JH1 domains is enriched in affected residues, since three of the JH1-located mutations are targeting the activation loop of the JH1 domain. The rest of the JH2-located mutations are spread around the domain without any interaction with the kinase domain, suggesting that these mutations might have an allosteric effect on the JH1-JH2 interaction.

For the mutations located in the JH1 kinase domain we took advantage of the recently solved crystal structure of the JAK1 JH1 domain in complex with JAK inhibitor CMP6 to describe their localization in more details (18). As shown in Figure 4C, from 8 JH1-located mutations, three (K1026E, Y1035C and S1043I) are located in the activation loop (A-loop), two mutations affect the same residue (F958V and F958C) in the hinge region of ATP-binding pocket, two others (D895H and T901R) are located at the top the kinase domain in the loop formed between two antiparallel β-strands (β2 and β3) and one mutation affects the loop formed between β3 β-strand and αC helix of JH1 domain.

Interestingly, one of the three mutations in the activation loop involves the Y1035 residue, which is the second tyrosine of the "KEYY" motif forming a twin tyrosine phosphorylation site (19). Autophosphorylation of the first of these tyrosines, Tyr1034 in JAK1, is critical for tyrosine kinase activation. The role of the second tyrosine is less clear (20,21). As shown in Figure 4D, the two affected residues K1026 and Y1035 are interacting in the wild- type JAK1 as also reported for the homologous residues K999 and Y1008 in JAK2 (22). Interestingly, in our *in vitro* autonomous selection, mutation of each of these residues (Figure 4D: K1026E/Y1035 or K1026/ Y1035C) renders JAK1 kinase constitutively active. This suggests that interaction between the two residues is important for the balance between the active and inhibited conformations of the JAK kinase and might represent another hotspot for activating mutations.

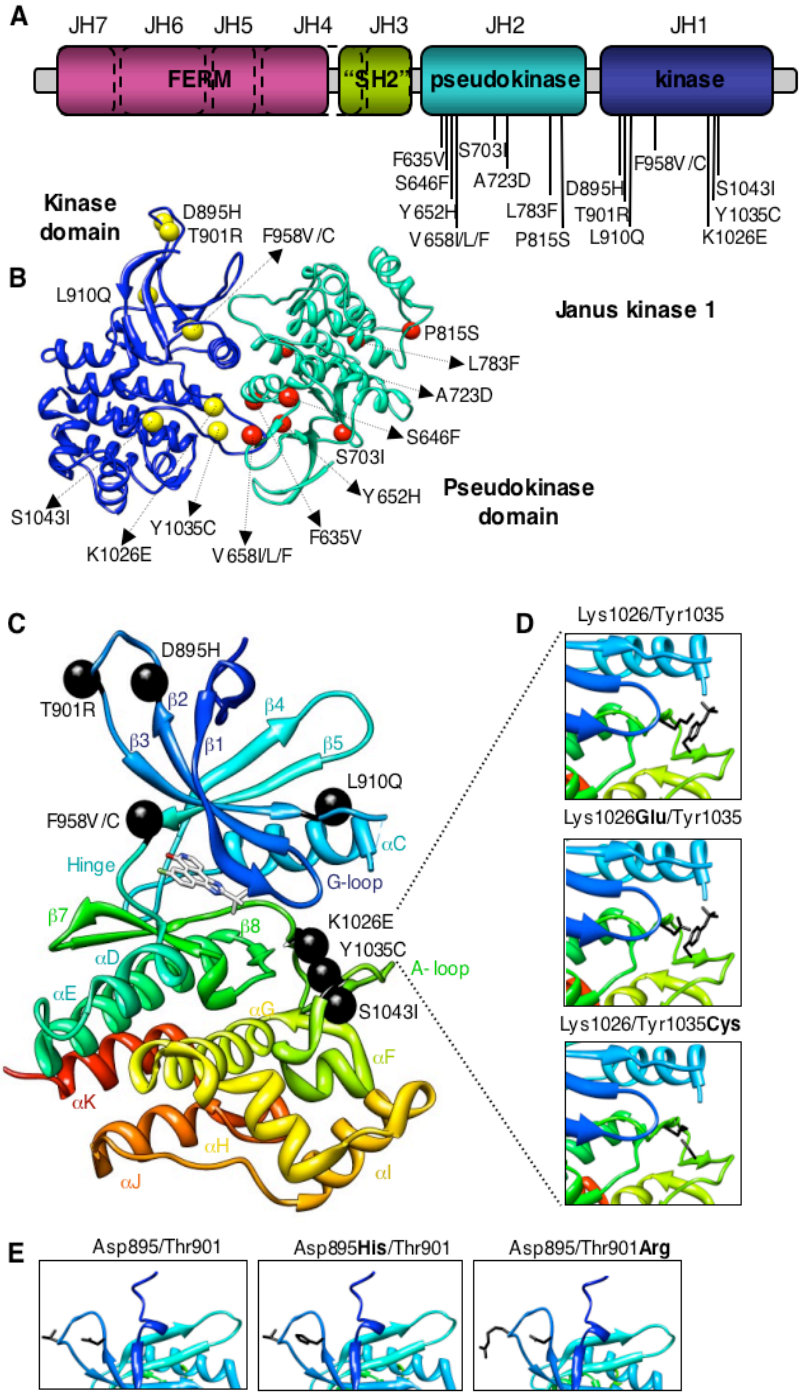

FIGURE 4 Spontaneous activating mutations define various potential hotspots in JAK1 structure. *A) Localization of 18 identified missense point mutations in the schematic structure of JAK1 with its functional domains and B) in the three-dimensional modeled structure of kinase and pseudokinase domain published by (12). 10 affected residues (red balls) in pseudokinase (in green) and 8 (yellow balls) in kinase domain (in blue) are shown. C) Localization of 8 affected residues (black balls) in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 in ribbon representation (18). The N-terminal lobe (residues 865-958) comprises a five-stranded antiparallel β-sheet (β1 to β5) and one α-helix (αC). The larger C-terminal lobe (residues 865-958) is predominantly helical with eight α-helices (αD to αK) with one main pair of antiparallel β-strands (β7-β8). Localizations of the hinge region, glycine loop (G-loop) and activation loop (A-loop) are indicated with the corresponding colors. The JAK inhibitor is presented in white using a ball-and-stick representation. D) Magnification of structure of the activation loop showing the side chains of the residues at positions 1026 and 1035 for the wild type JAK1 (top panel), the K1026E mutant (middle panel), and the Y1035C mutant (lower panel). E) Magnification of the β2/β3 linking loop highlighting the side chains of the residues at positions 901 and 958 for the wild type JAK1 (left panel), the Asp895His mutant (middle panel) and the Thr901Arg mutant (right panel).*

The last two mutations (D895H and T901R) are located at the top of the kinase domain (Figure 4C), far away from the key functional regions of the kinase, such as the ATP-binding pocket, catalytic loop or activation loop. Although the mechanism by which these residues might regulate the kinase activity remains unclear, their localization within the structure is close enough to allow for interaction between them and other surrounding residues within the loop and indicates that this loop could represent another hotspot for activating mutations in JAK1 (Figure 4D).

Co-expression of JAK1-binding receptors promotes JAK1 mutants-mediated constitutive STAT activation. Previously we showed that ALL-associated JAK1 mutants needed to associate to JAK1 binding receptors, such as IL-2R or IL-9R, to promote constitutive activation of the receptor complex and signal transduction via STAT transcription factors (23). We also showed that beside the classical IL-9 receptor complex (composed of γ chain, IL-9Rα and JAK3), IL-9Rα alone forms homodimeric complexes (24), which provide sufficient scaffold for JAK1 mutant-mediated constitutive STATs activation. Therefore we tested the ability of IL-9Rα and IL-2Rβ, alone or in complex, to promote the constitutive activation of STATs induced by our new JAK1 mutants. For that purpose, we used HEK293 cells, which do not express IL-9Rα, IL-2Rβ, γc or JAK3. As shown in Figure 5A for the first 13 of our mutants, while expression of JAK1 mutants alone did not result in constitutive STAT3 activation, co-expression of the IL-9Rα significantly increased STAT3 constitutive activation mediated by the JAK1 mutants. Similar results were obtained with the co-expression of γc and JAK3, which restores the full IL-9R complex, and for the activation of STAT5 with IL-2Rβ (Figure 5B). Taken together, these results confirm that all JAK1 mutants need to associate with a cytokine receptor to constitutively activate STAT factors.

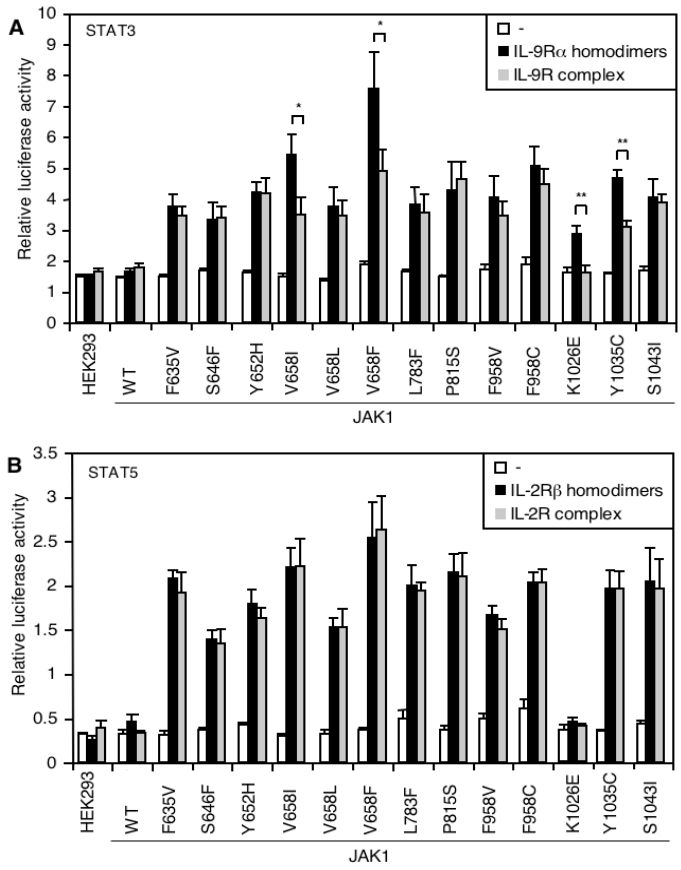

FIGURE 5 Co-expression of JAK1-binding receptors promotes JAK1 mutant-mediated constitutive STAT activation. *A) HEK293 cells were co-transfected with the cDNAs encoding wild type (WT) or mutated JAK1, IL-9Rα with and without γc and JAK3, in addition to the STAT3-responsive luciferase reporter construct pGL3-pap1. 24 hours post-transfection, cells were subjected to a luciferase assay. Results are mean ± SEM from 4 independent experiments. B) The same experiments were performed with IL-2Rβ and STAT5-responsive luciferase reporter pLHRE. Results are mean ± SEM from 3 independent experiments.*

Anti-proliferative effect of type I IFN on autonomous clones with spontaneous activating mutations in JAK1. Type I IFNs are known to exert anti-proliferative activity on various cell types. Previously, we showed that blasts from JAK1 mutation-positive ALL patients are characterized by a type I IFN transcriptional signature, and that some ALL-associated JAK1 mutants, namely A634D and R724H, potentiate the anti-proliferative effect of type I IFNs *in vitro* and *in vivo*, while the R879C mutation did not (25). Our *in vitro* model of tumorigenesis provides us with murine autonomous clones heterozygous for activating mutations already identified in patients, but also clones expressing activating JAK1 mutations that have not been described yet. We took advantage of this system to test the ability of these new JAK1 mutants to potentiate the cellular response to IFNs. As shown in Figure 6, 13 out of the 18 JAK1 mutants (F958V, Y1035C, P815S, F958C, S1043I, S646F, Y652H, F635V, V658F, L783F, S703I, K1026E, L910Q, D985H, T901R and A723D), representing 72% of the tested mutations, increased the sensitivity of BaF3 cells to the anti-proliferative effect of IFNα when compared to control parental cells (*$p<0.05$, **$p<0.01$).

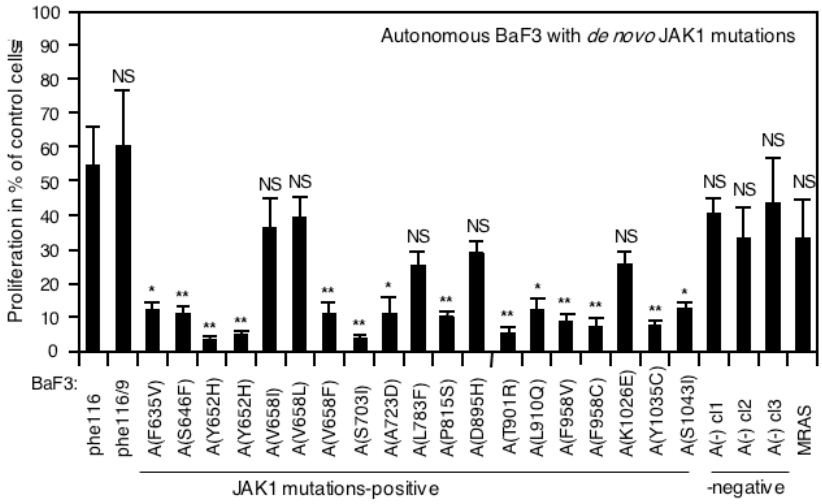

FIGURE 6 Anti-proliferative effect of type I IFN on autonomous clones with spontaneous activating mutations in JAK1. *4,000 control parental BaF3 phe116, phe116/9, 19 JAK1 mutation-positive and 3 JAK1 mutation-negative autonomous cells were seeded in 96-well plates and stimulated by 1% IFN-α or control HEK293 SN. After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results correspond to the proliferation of cells in the presence of IFN-α presented as the mean percentage relative to control cultures ± SEM from at least 3 independent experiments performed in triplicates. Dunnett multiple comparisons test was used to determine which clones were significantly more sensitive to the anti-proliferative effect of IFN-α (* P<0.05; ** P<0.01) as compared to the parental phe116 BaF3 cells.*

As previously observed with the human ALL-associated R879C JAK1 mutant, some mutations (V658I/L, K1036E, L783F and D895H) did not significantly alter the IFN activity. The results were reproducible because several independent autonomous clones harboring the same mutation in JAK1 displayed similar sensitivity to IFN-α, as illustrated for BaF3 A(Y652H) in Figure 6. Noticeably, like autonomous BaF3 cells transfected with a mutated MRAS isoform, autonomous BaF3 clones negative for JAK1 mutations, in which unknown genetic alterations are responsible for their autonomous proliferation, were not hypersensitive to IFN-α.

Activating JAK1 mutations F958V, F958C and K1026E confer resistance to JAK inhibitor I.

We took advantage of our collection of JAK1 mutant-induced autonomously proliferating clones to test their sensitivity to a specific JAK inhibitor. As shown in Figure 7A, in contrast to BaF3 MRAS, an autonomous BaF3 cell with constitutive MAPK activation, treatment with 1 μM of JAK inhibitor I for 3 days completely blocked proliferation of the majority of JAK1 mutation-dependent cell lines with three exceptions. BaF3 cell lines heterozygous for F958V, F958C or K1026 JAK1 mutations showed a significant residual proliferation in the presence of JAK inhibitor I. Long term treatment showed that these cells were able to growth in the presence of high concentration of JAK inhibitor I (1 μM), indicating that the JAK1 mutants expressed in these clones are resistant to the ATP-mimicking inhibitor (data not shown). We

further evaluated the activity of the different JAK1 mutants after treatment with JAK inhibitor I by assessing tyrosine phosphorylation of STAT5. A short-term treatment of the autonomous clones A(V658I), but not of the A(F958V) or A(F958C) cells with 1 μM JAK inhibitor I, completely blocked STAT5 phosphorylation (Figure 7B), showing that the F958V mutant is resistant to JAK inhibition. Similar results were obtained in BaF3 cells stably transduced with the different mutants, as shown in Figure 7C.

The impact of the F958V and F958C mutations on the relative positions of this mutated residue and ATP-competitive inhibitors is highlighted by the crystal structure of the JH1 domain with the CMP6 inhibitor. As shown in Figure 7D, the phenylalanine residue at position 958 is in direct interaction with the inhibitor through its main-chain atoms, as previously described (18). In this model, replacement of Phe at position 958 by Val and Cys abolishes such an interaction as the orientation of these two relatively small amino acids is opposing the orientation of phenylalanine at the same position (see Figure 7D, Phe958Val and Phe958Cys). As a result, the Val and Cys residues at position 958 also render the ATP-binding pocket somehow more “open” and thus accessible for ATP than with the original Phe residue, suggesting that this could be the mechanism leading to constitutive kinase activity of the JAK1 F958V and F958C mutants.

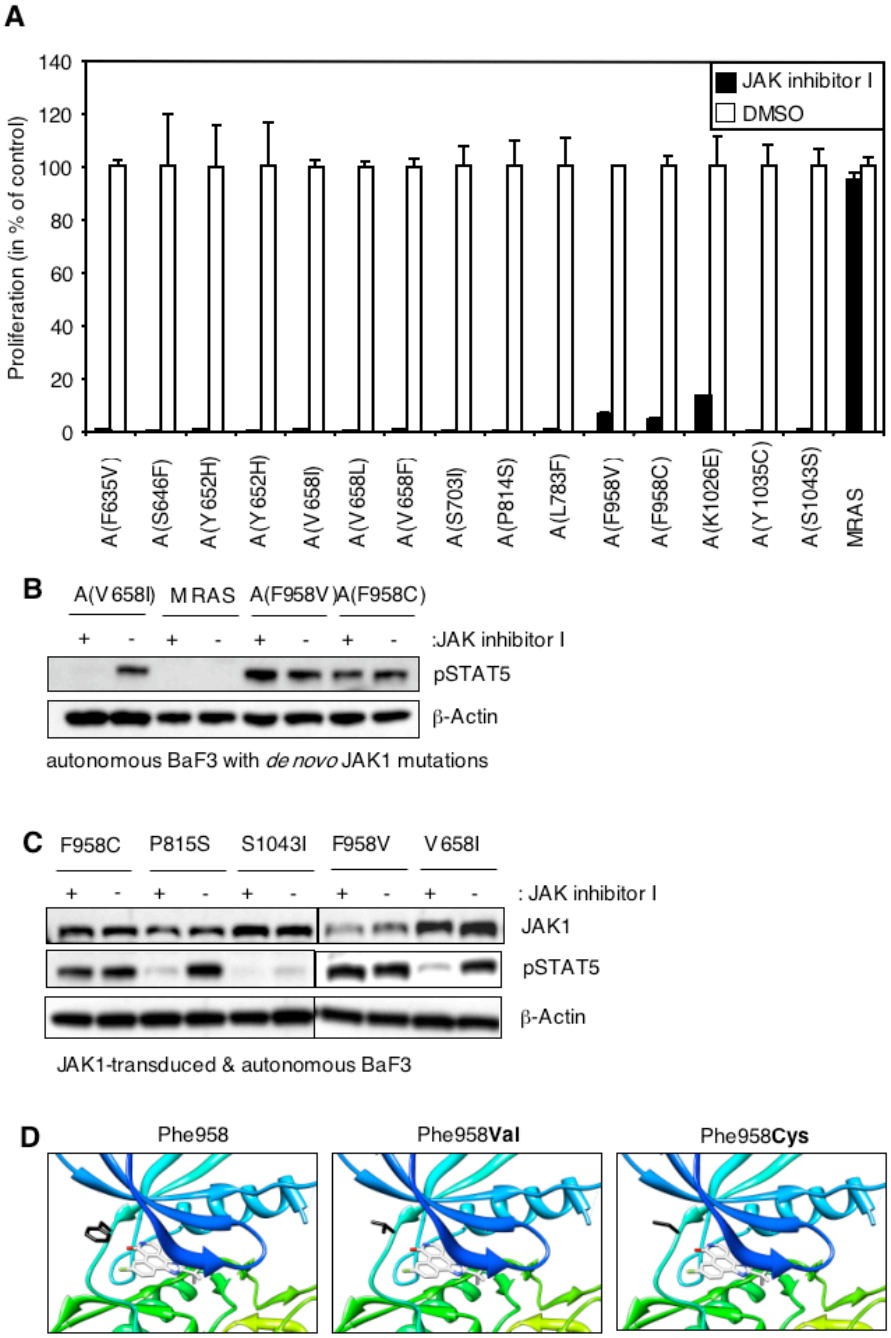

FIGURE 7 Activating JAK1 mutations F958V, F958C and K1026E confer resistance to JAK inhibitor I. *A) 20,000 autonomous BaF3 heterozygous for different JAK1 mutations or transfected with an activated MRAS mutant (BaF3 MRAS) were seeded in 96-well plates with 1mM JAK inhibitor I or DMSO. After 24 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results correspond to the mean ± SD of cell culture triplicates. Similar results were obtained in 3 independent experiments. B) 10^6 autonomous BaF3 A(V658I), A(F958V), A(K1026E) heterozygous for JAK1 mutations and BaF3 MRAS cells were treated for 15 min with 1 μM JAK inhibitor I or DMSO. Cells were then lyzed and subjected to Western Blot analysis. Basal phosphorylation of STAT5 was detected using specific anti-pY694 STAT5 antibody. Membranes were reprobed with anti-JAK1 and anti-β-actin antibodies as a control. Similar results were obtained in 3 independent experiments. C) The same experiment was performed using autonomous BaF3 cells stably transduced with F958V, F958C, V658I, P815S and S1043I JAK1 mutants. Similar results were obtained in 3 independent experiments. D) Magnification of the ATP-binding lobe in the crystal structure of JAK1 kinase domain solved with ATP-competitive JAK inhibitor CMP6 (18) highlighting the side chains of the residue 958 for the wild type JAK1 (left panel), the Phe958Val mutant (middle panel) and the Phe958Cys mutant (right panel).*

DISCUSSION

The main observation reported here is that a broad spectrum of spontaneous mutations in one of the endogenous JAK1 alleles is the main mechanism, that secondary to the overexpression of the JAK1 transcript, confer cytokine-independent growth capability *in vitro* and full tumorigenicity *in vivo* (7). The most common explanation for the acquisition of genetic abnormalities in cancer is based on a 'natural selection' process, where clones that have a selective advantage because of the random acquisition of a favorable mutation will outgrow the rest of the population. The most striking observation of our model is the fact that 80% of the autonomous clones harbor a mutation in JAK1, while many other genetic hit would be expected to promote the autonomous growth of BaF3, including mutations in RAS, JAK2, JAK3, translocations between TEL and JAK2 or BCR and ABL1, and many others.

Several hypotheses can explain this phenomenon. First, a simple explanation would be that mutations occur randomly in such proto-oncogenes in the cell culture, but that the normal expression level of these proteins is not sufficient for a mutant in one single amino acid to exhibit its full transformation effect to promote cytokine-independent growth of BaF3 cells. Thus activating mutations might also occur in the parental BaF3 cells, but these would not provide any selective advantage unless JAK1 expression is increased, as this happen in our model resulting from the first step of selection.

Alternatively, we could speculate that the mutations occurring in our model are not random and accumulate specifically in the JAK1 gene. Indeed, in recent years, transcription has proven to participate in the induction of genetic instability and diversity. It was shown that mutations in a particular gene could be induced by increase in transcription and the process was coined transcription-associated mutations (TAM). Although the exact mechanism behind this phenomenon is not known, several hypotheses have been designed based on the fact that DNA replication, repair and recombination can virtually occur in a DNA substrate that is simultaneously transcribed. However, it is worth noting that one example of TAM represents starvation-induced also called "adaptive" mutations. Adaptive mutation stands for the increase genome-wide mutation rate, affecting however genes of selected function and is associated with increased expression of affected genes during prolong stress in cells that are not dividing (26). Finally, we can imagine a mechanism of 'oncogene addiction' to the IL-9R-JAK-STAT pathway in the BaF3 phe116/9 cells. A mutation in the kinase of that pathway, such as in JAK1, will maintain the activation of the pathway after cytokine deprivation, while in other cases, cells will die of an 'oncogenic shock' (27) even if other mutations are present.

We can not exclude either that combination of these and other processes is involved in accumulation of mutations in JAK1 gene in our model.

In our *in vitro* model, we identified 18 spontaneously acquired point mutations in JAK1, of which 14 are newly described, while 4 have been previously reported in human patients. All the mutations that were functionally studied are activating because their transfection into the cytokine-dependent BaF3 cells induced autonomous growth and constitutive activation of the JAK-STAT pathway. 10 of these mutations affect residues from the pseudokinase domain and the 8 other mutations target the kinase domain of JAK1. Mutations in the pseudokinase domain include point mutations of the Val658 residue to three different amino acids – isoleucine (I), leucine (L) and phenylalanine (F). The latter mutation was described in human T-ALL blasts (13). These three JAK1 mutations were predicted to be activating because the V658 residue corresponds to the conserved residue V617 of JAK2, which is frequently mutated to phenylalanine in myeloproliferative neoplasms, and because V617L and V617I also activate JAK2 (28). This residue is located at an interface between the kinase and the pseudokinase domains of JAKs, and is supposed to contribute to the inhibition of the kinase activity by the pseudokinase domain.

Two other mutations, described both in T-ALL patients and in our model, target residues adjacent to V658 and could share a similar mechanism of kinase activation: S646F and Y652H. In the same interface between the kinase and pseudokinase domains, the F635V mutation described here is a novel mutation, but the A634D mutation affects the proceeding residue in T-ALL patients (12), further pointing to this region as a key interface for the regulatory activity of the pseudokinase domain. Among the four other mutations of the pseudokinase domain that appear to be scattered throughout the pseudokinase domain, the L783F mutant has been previously described in T-ALL and AML patients by two independent groups (13,29), further stressing the relevance of our model for human malignancies. In this line, the activating mutation A723D described here is adjacent to the R724H mutation that we described in T- and B-ALL patients (12,30). The JAK2 mutations R683S/G/K that affect the homologous residue to JAK1 R724 has been reported in Down syndrome and pediatric B-ALL patients (31), and a deletion of the surrounding motif found in Down syndrome B cell precursor-ALL patients renders JAK2 constitutively active (32). As the majority of the mutated residues identified here are conserved between all the members of the JAK family (JAK1, JAK2, JAK3 and TYK2). We can therefore predict that homologous mutations in the other members of the family will also be activating, and could be found in malignancies.

So far, mutation of only one residue Arg 879 in the kinase domain of JAK1 has been reported (12). Here we describe 8 novel mutations in this domain. The K1026E, Y1035C and S1043I mutants target the activation loop of the kinase domain. The Y1035 residue is the second tyrosine of the "KEYY" motif forming a twin tyrosine phosphorylation site within the activation loop (19). Autophosphorylation of the first of these tyrosines, Tyr1034/Tyr1007 in JAK1/JAK2, is critical for tyrosine kinase activation. The role of the second tyrosine is less clear (20). In JAK3, substitution of the second tyrosine by phenylalanine resulted in increased kinase activity (21), suggesting that phosphorylation of this residue might have an inhibitory effect on kinase activity of JAKs. Interestingly, recently solved crystal structure of the JAK2 kinase domain shows that in active conformation both tyrosines are stabilized by interaction with several lysine residues. Importantly, the second tyrosine, Tyr1008 in JAK2, is stabilized by the single Lys999 residue, which is conserved within the JAK family members. The two interacting residues, the Lys999 and Tyr1008 of JAK2, are homologous to residues Lys1026 and Tyr1035 in JAK1, both mutated in our model.

JAK inhibition becomes part of the treatment of MPN patients harboring mutations in JAK2, and some of these ATP-competitive JAK2 inhibitors show a very good 'off-target' inhibition of JAK1. Such inhibitors represent good candidates to treat patients harboring mutations in JAK1. It was therefore interesting to test the sensitivity of the different mutants to ATP-competitive JAK inhibitors to detect mutations resistant to the inhibition. Similar screenings were performed in BaF3 cells to predict mutations resistant to the inhibition of BCR-ABL, which was confirmed in the clinic in chronic myeloid leukemia patients (33). While all the pseudokinase mutants remain sensitive to JAK inhibition, three kinase domain mutants were resistant: F958V, F958C and K1026E. The residue Phe958 is located in the hinge region of ATP binding pocket in the kinase domain. Recently the crystal structures of the JAK1 kinase domain in complexes with two pan-JAK inhibitors (CMP6 and CP-690, 550) were solved. These structures showed high similarities in the binding mode of the two ATP-competitive JAK inhibitors revealing Glu883 in the glycine loop and Phe958 in hinge region as unique and crucial residues that make direct contacts with these inhibitors through their main-chain atoms (18). Here we show that Phe958 residue is not only important for the inhibitory capacity of ATP-competitive JAK inhibitors, but that its change to valine or cysteine renders JAK1 kinase constitutively active. Consequently, patients positive for these activating mutations would be resistant to ATP-competitive JAK inhibitors. Interestingly, the K1026E mutation, localized outside the ATP binding pocket was also resistant to JAK inhibitors.

Finally, we show here that most, but not all, JAK1 activating mutants confer hypersensitivity to the anti-proliferative effect of type I IFN, confirming our initial observations with JAK1 mutants found in human ALL patients. This property could not be associated with a particular hotspot of mutations but appeared to be reproducible for independent clones exhibiting the same mutation, indicating that the IFN hyperresponsiveness is intrinsically linked to the JAK1 mutation. Although the IFN treatment remains an attractive possibility for ALL patients with JAK1 mutation, the heterogeneity of the *in vitro* response might be an issue, as also suggested by the potential JAK inhibitor resistance described above. However, the common characteristic apparently shared by all JAK1 mutants is the fact that they exert their transforming activity only when expressed beyond the regular level, which suffices for normal cytokine receptor signaling. In this respect, we believe that elucidating the regulatory mechanisms that underlie JAK1 overexpression in our model might lead to new perspectives to modulate the function of pathogenic mutants.

MATERIALS AND METHODS

Cell culture and cytokines. BaF3 mouse hematopoietic pro-B cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and IL-3 (150 U/ml), which was produced by transfected CHO cells. The generation of BaF3 cells transfected with the mutated hIL-9 receptor Y116F (BaF3 phe116) as well as selection of IL-9 responsive BaF3 phe116/9 cells was previously described (7). Recombinant human IL-9 was produced in the baculovirus system and purified by affinity chromatography in our laboratory. The frequency of autonomous cells was assessed as previously described (7). IL-9-selected BaF3 phe116/9 and non-selected BaF3 phe116 cells were grown in the presence of IL-3. Cells were then washed twice in PBS pH7.4, resuspended at 5 x 10^5/ml in culture medium without IL-3, and cultured in 96-well microtiter plates at 5 x 10^4 cells/ well. Plates were checked for proliferating clones during three weeks. Clones were subsequently cultured and amplified in the same conditions as those described for BaF3 cells but without IL-3. HEK293 human embryonic kidney 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.

RNA extraction, cDNA synthesis, PCR and sequencing. Total RNA was isolated from 10^6 IL-3 dependent BaF3 phe116, BaF3 phe116/9 or autonomous BaF3 clones using TriPure reagent (Roche) according to the manufacturer's instructions. Reverse transcription was performed on 1 µg of total RNA with an oligo (dT) primer (Roche) and M-MLV RT (Invitrogen). PCR

amplification was performed from cDNA corresponding to 20 ng of total RNA at 94°C for 1 min, 58 °C for 1 min, and 72°C for 2 min with total number of cycles of 30. Depending on the distinct region of JAK1 desired to sequence different sets of primers were used to amplify and sequence JAK1 PCR product (available upon request).

After size verification of each PCR product in 1% agarose gel with ethidium bromide, 50 µl of PCR product was purified using Chromaspin technology (Clontech). Nanodrop (Thermo Scientific) was used to quantify the concentration of the purified PCR product. 50-100 ng of PCR product was used to sequence with the DYEnamic ET Dye Terminator Kit (Amersham Biosciences, Freiburg, Germany) according to manufacturer's instructions. Two independent PCR reactions were performed to rule out possibility of Taq-induced mutations during amplification. The numbering of mutated amino acids was based on the human JAK1 sequence, allowing to remain consistent with currently used nomenclature for JAK1 protein mutations in literature and to localize our JAK1 mutations in the previously described three-dimensional structure model of human JAK1 protein (12).

Plasmid construction, stable DNA transfections and analysis of transfected cells. All JAK1 mutants (P815S, F635V, S646F, Y652H, V658F, V658L, V658I, S703I, L783F, F958C, F958V, K1026E, Y1035C, S1043I) were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene, La Jolla, California) and subcloned into the pMX-GFP biscistronic retroviral vector upstream of the IRES as previously described (12,23). The mutated JAK1 constructs were verified by sequencing of the full-length JAK1 sequence using DYEnamic ET Dye Terminator Kit (Amersham Biosciences). For stable transduction, typically, 0.5 x 10^6 BaF3 cells were infected by retroviruses produced by BOSC packaging cells using standardized protocol previously described (34). Cells were subsequently sorted by FACS for equal level of GFP. Human IL-9Rα and IL-9Rα Y116F (phe116) were generated as previously described (6).

Dual luciferase assay. STAT3 and STAT5 transcriptional activity was assessed by measurements of luciferase expression in HEK293 cells upon transient transfection of appropriate cDNA constructs and pGL3-pap1-luc or pLHRE-luc reporter vectors. Another reporter plasmid, *Renilla* luciferase (pRLTk, Promega), was co-transfected as an internal transfection control. STAT5-mediated transcription was evaluated with the pLHRE-luc reporter gene constructs harboring tandem copies of the STAT5-inducible lactogenic hormone response element (LHRE) of the rat β-casein gene promoter, inserted upstream a

luciferase gene (35). STAT3-mediated transcription was evaluated with pGL3-pap1-luc plasmid containing the luciferase gene under the control of the STAT3-inducible rat *Pap1* (pancreatitis-associated protein-1) promoter (36). Transient transfection of HEK293 cells by LipofectAMINE (Invitrogen, Merelbeke, Belgium) was previously described (23). Briefly, HEK293 cells were seeded in 24-well plates at $2x10^5$ cells/well one day before transfection. Transfection was carried out according to the manufacturer's recommendations, using 200 ng of the appropriate constructs, 250 ng of pGL3-pap1 or pLHRE reporter plasmids, 50 ng of pRLTk control plasmid and empty vector to normalize total transfected DNA to 1 µg. 24 hours after transfection, cells were lysed by 150 µl of Passive Lysis Buffer 1x supplied by Promega. Luciferase assays were performed using the dual luciferase reporter assay kit (Promega, Madison, WI).

Western blots. For Western Blot analysis, 10^6 BaF3 cells were starved for 4 hours and then collected and lysed in 250 µl of Laemmli buffer (BioRad, Hercules, CA) using a 20-gauge syringe. Autonomous BaF3 cells that were grown in the absence of cytokine were directly collected and lysed. For the JAK inhibition experiments, 10^6 BaF3 autonomous cells were stimulated with 1 mM of JAK inhibitor I (Calbiochem), or DMSO for 15 minutes. Cellular lysates were boiled for 3 minutes before loading 20 µl on 12% pre-cast Tris-Glycine gels (Invitrogen, Merelbeke, Belgium) and electrophoretically transferred to nitrocellulose membranes (Hybond-C; Amersham, Buckingham, England). After blocking of the membranes in 5% milk/TBS-Tween, immunoblotting was performed by overnight incubations at 4°C with primary antibodies in 0.1% TBS-Tween, using the dilution recommended by the manufacturer. After 3 x 5 minutes of washing in 0.1% TBS-Tween, the membranes were incubated with secondary anti-rabbit-HRP or anti-mouse-HRP antibodies from Cell Signaling Technology (Beverly, MA), diluted 1/5,000 in 5% PBS-TBS-Tween. After 3 x 5 minutes of washing, a SuperSignal West Pico detection kit (Pierce, Rockford, IL) and Kodak Biomax Light Film were used for detection. Phosphorylation of signaling proteins was investigated using the following phospho-specific antibodies from Cell Signaling Technology: anti-pY1034/1035 JAK1 (#3331S), anti-pY705 STAT3 (#9131S), anti-pY694 STAT5 (#9351S) and anti-p44/42 MAPK (#9101L). Blots were re-probed with anti-JAK1 (#3332) (Cell Signaling Technology, Beverly, MA), anti-STAT5 (Santa Cruz) or anti-β-actin (Sigma) antibodies, as a control.

Cytokine-independent proliferation assay. Stably-transduced BaF3 cells were washed three-

times in PBS and seeded in 24-well plates at a concentration of 10,000 cells/ml without mIL-3. As control, 150 U/ml of IL-3 was added to the BaF3 cell cultures to check the proper proliferation of each cell line. After 72 hours, living cells were counted in both conditions, with or without mIL-3. Triplicate cultures were generated to calculate mean values and SD.

The type I IFN proliferation assay. 5,000 stably transduced BaF3 cells were seeded in 96-well plates and stimulated by 1% of murine IFN-α11 or mock/control HEK293 supernatant. All experiments were performed in the presence of IL-3. After 72 hours, tritiated thymidine was added to the cells for 4 hours. Cells were then collected on microfiltered plates, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard, Meriden, CT).

JAK1 structures. Three-dimensional structural model of the kinase and pseudokinase domain was the previously described model of JAK1 (12), obtained by employing Deep View software and the Swiss Model server after manual optimalization of the alignment. Structural data from recently solved crystal structure of JAK1 kinase domain in complex with CPM, an ATP-competitive inhibitor (18), were used to analyze more precisely the impact of the kinase domain-located JAK1 mutations. Molecular graphics images were produced using the UCSF Chimera package from the Resource for Biocomputing, Visualization, and Informatics at the University of California, San Francisco (37).

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REFERENCES

1. Hanahan, D., and Weinberg, R. A. (2000) *Cell* **100**, 57-70
2. Van Etten, R. A. (2007) *Oncogene* **26**, 6738-6749
3. Hallek, M., Bergsagel, P. L., and Anderson, K. C. (1998) *Blood* **91**, 3-21
4. Bataille, R., Barlogie, B., Lu, Z. Y., Rossi, J. F., Lavabre-Bertrand, T., Beck, T., Wijdenes, J., Brochier, J., and Klein, B. (1995) *Blood* **86**, 685-691

5. Knoops, L., and Renauld, J. C. (2004) *Growth Factors* **22**, 207-215
6. Demoulin, J. B., Uyttenhove, C., Van Roost, E., DeLestre, B., Donckers, D., Van Snick, J., and Renauld, J. C. (1996) *Mol Cell Biol* **16**, 4710-4716
7. Demoulin, J. B., Uyttenhove, C., Lejeune, D., Mui, A., Groner, B., and Renauld, J. C. (2000) *Cancer Res* **60**, 3971-3977
8. Knoops, L., Hornakova, T., Royer, Y., Constantinescu, S. N., and Renauld, J. C. (2008) *Oncogene* **27**, 1511-1519
9. Schindler, C., Levy, D. E., and Decker, T. (2007) *J Biol Chem* **282**, 20059-20063
10. Haan, C., Is'harc, H., Hermanns, H. M., Schmitz-Van De Leur, H., Kerr, I. M., Heinrich, P. C., Grotzinger, J., and Behrmann, I. (2001) *J Biol Chem* **276**, 37451-37458
11. Wilks, A. F. (2008) *Semin Cell Dev Biol* **19**, 319-328
12. Flex, E., Petrangeli, V., Stella, L., Chiaretti, S., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., Martinelli, S., Fragale, A., Sanchez, M., Tavolaro, S., Messina, M., Cazzaniga, G., Camera, A., Pizzolo, G., Tornesello, A., Vignetti, M., Battistini, A., Cave, H., Gelb, B. D., Renauld, J. C., Biondi, A., Constantinescu, S. N., Foa, R., and Tartaglia, M. (2008) *J Exp Med* **205**, 751-758
13. Jeong, E. G., Kim, M. S., Nam, H. K., Min, C. K., Lee, S., Chung, Y. J., Yoo, N. J., and Lee, S. H. (2008) *Clin Cancer Res* **14**, 3716-3721
14. Mullighan, C. G., Zhang, J., Harvey, R. C., Collins-Underwood, J. R., Schulman, B. A., Phillips, L. A., Tasian, S. K., Loh, M. L., Su, X., Liu, W., Devidas, M., Atlas, S. R., Chen, I. M., Clifford, R. J., Gerhard, D. S., Carroll, W. L., Reaman, G. H., Smith, M., Downing, J. R., Hunger, S. P., and Willman, C. L. (2009) *Proc Natl Acad Sci U S A* **106**, 9414-9418
15. Lindauer, K., Loerting, T., Liedl, K. R., and Kroemer, R. T. (2001) *Protein Eng* **14**, 27-37
16. James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., and Vainchenker, W. (2005) *Nature* **434**, 1144-1148
17. Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber, W. N., and Green, A. R. (2005) *Lancet* **365**, 1054-1061
18. Williams, N. K., Bamert, R. S., Patel, O., Wang, C., Walden, P. M., Wilks, A. F., Fantino, E., Rossjohn, J., and Lucet, I. S. (2009) *J Mol Biol* **387**, 219-232
19. Hubbard, S. R., and Till, J. H. (2000) *Annu Rev Biochem* **69**, 373-398
20. Feng, J., Witthuhn, B. A., Matsuda, T., Kohlhuber, F., Kerr, I. M., and Ihle, J. N. (1997) *Mol Cell Biol* **17**, 2497-2501
21. Zhou, Y. J., Hanson, E. P., Chen, Y. Q., Magnuson, K., Chen, M., Swann, P. G., Wange, R. L., Changelian, P. S., and O'Shea, J. J. (1997) *Proc Natl Acad Sci U S A* **94**, 13850-13855
22. Lucet, I. S., Fantino, E., Styles, M., Bamert, R., Patel, O., Broughton, S. E., Walter, M., Burns, C. J., Treutlein, H., Wilks, A. F., and Rossjohn, J. (2006) *Blood* **107**, 176-183
23. Hornakova, T., Staerk, J., Royer, Y., Flex, E., Tartaglia, M., Constantinescu, S. N., Knoops, L., and Renauld, J. C. (2009) *J Biol Chem* **284**, 6773-6781
24. Malka, Y., Hornakova, T., Royer, Y., Knoops, L., Renauld, J. C., Constantinescu, S. N., and Henis, Y. I. (2008) *J Biol Chem* **283**, 33569-33577
25. Hornakova, T., Chiaretti, S., Lemaire, M. M., Foa, R., Ben Abdelali, R., Asnafi, V., Tartaglia, M., Renauld, J. C., and Knoops, L. *Blood*
26. Aguilera, A. (2002) *Embo J* **21**, 195-201

27. Sharma, S. V., Fischbach, M. A., Haber, D. A., and Settleman, J. (2006) *Clin Cancer Res* **12**, 4392s-4395s
28. Dusa, A., Staerk, J., Elliott, J., Pecquet, C., Poirel, H. A., Johnston, J. A., and Constantinescu, S. N. (2008) *J Biol Chem* **283**, 12941-12948
29. Xiang, Z., Zhao, Y., Mitaksov, V., Fremont, D. H., Kasai, Y., Molitoris, A., Ries, R. E., Miner, T. L., McLellan, M. D., DiPersio, J. F., Link, D. C., Payton, J. E., Graubert, T. A., Watson, M., Shannon, W., Heath, S. E., Nagarajan, R., Mardis, E. R., Wilson, R. K., Ley, T. J., and Tomasson, M. H. (2008) *Blood* **111**, 4809-4812
30. Asnafi, V., Le Noir, S., Lhermitte, L., Gardin, C., Legrand, F., Vallantin, X., Malfuson, J. V., Ifrah, N., Dombret, H., and Macintyre, E. (2009) *Br J Haematol*
31. Bercovich, D., Ganmore, I., Scott, L. M., Wainreb, G., Birger, Y., Elimelech, A., Shochat, C., Cazzaniga, G., Biondi, A., Basso, G., Cario, G., Schrappe, M., Stanulla, M., Strehl, S., Haas, O. A., Mann, G., Binder, V., Borkhardt, A., Kempski, H., Trka, J., Bielorei, B., Avigad, S., Stark, B., Smith, O., Dastugue, N., Bourquin, J. P., Tal, N. B., Green, A. R., and Izraeli, S. (2008) *Lancet* **372**, 1484-1492
32. Malinge, S., Ben-Abdelali, R., Settegrana, C., Radford-Weiss, I., Debre, M., Beldjord, K., Macintyre, E. A., Villeval, J. L., Vainchenker, W., Berger, R., Bernard, O. A., Delabesse, E., and Penard-Lacronique, V. (2007) *Blood* **109**, 2202-2204
33. Shah, N. P., Tran, C., Lee, F. Y., Chen, P., Norris, D., and Sawyers, C. L. (2004) *Science* **305**, 399-401
34. Seubert, N., Royer, Y., Staerk, J., Kubatzky, K. F., Moucadel, V., Krishnakumar, S., Smith, S. O., and Constantinescu, S. N. (2003) *Mol Cell* **12**, 1239-1250
35. Gerland, K., Bataille-Simoneau, N., Basle, M., Fourcin, M., Gascan, H., and Mercier, L. (2000) *Mol Cell Endocrinol* **168**, 1-9
36. Peelman, F., Iserentant, H., De Smet, A. S., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2006) *J Biol Chem* **281**, 15496-15504
37. Pettersen, E. F., Goddard, T. D., Huang, C. C., Couch, G. S., Greenblatt, D. M., Meng, E. C., and Ferrin, T. E. (2004) *J Comput Chem* **25**, 1605-1612

Somatically Acquired JAK1 Mutations in Adult Acute Lymphoblastic Leukemia

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Preamble

Having shown that JAK1 mutations induce tumoral transformation in an *in vitro* mouse model, we wanted to address the putative role of such mutations in human tumors.

In fact, this part of the work was performed in parallel with the project described above and was initiated by professor Marco Tartaglia from the University of Rome. This group collected samples from adult and pediatric patients with B- and T-cell acute lymphoblastic leukemia (B-ALL and T-ALL) and identified several point mutations in JAK1 in such leukemic cells.
My contribution to this work consisted in expressing and studying the activity of these JAK1 mutants in two cellular systems: in BaF3 cells, a murine IL-3 dependent pro-B cell line and in BW5147 cells, a murine leukemic cell line.

In this paper, we show that the A634D and R724H mutations induce the autonomous growth of the cytokine-dependent BaF3 cell line, and that the A634D and R879C JAK1 mutants protect leukemic BW5147 cells against dexamethasone-induced apoptosis, indicating that these three mutations among all the identified mutations in JAK1 gene are gain-of-function mutations that participate in the leukemogenic process by inducing the constitutive activation of the JAK-STAT pathway.

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Somatically acquired *JAK1* mutations in adult acute lymphoblastic leukemia

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Aberrant signal transduction contributes substantially to leukemogenesis. The *Janus kinase 1* (*JAK1*) gene encodes a cytoplasmic tyrosine kinase that noncovalently associates with a variety of cytokine receptors and plays a nonredundant role in lymphoid cell precursor proliferation, survival, and differentiation. We report that somatic mutations in *JAK1* occur in individuals with acute lymphoblastic leukemia (ALL). *JAK1* mutations were more prevalent among adult subjects with the T cell precursor ALL, where they accounted for 18% of cases, and were associated with advanced age at diagnosis, poor response to therapy, and overall prognosis. All mutations were missense, and some were predicted to destabilize interdomain interactions controlling the activity of the kinase. Three mutations that were studied promoted JAK1 gain of function and conferred interleukin (IL)-3–independent growth in Ba/F3 cells and/or IL-9–independent resistance to dexamethasone-induced apoptosis in T cell lymphoma BW5147 cells. Such effects were associated with variably enhanced activation of multiple downstream signaling pathways. Leukemic cells with mutated *JAK1* alleles shared a gene expression signature characterized by transcriptional up-regulation of genes positively controlled by JAK signaling. Our findings implicate dysregulated JAK1 function in ALL, particularly of T cell origin, and point to this kinase as a target for the development of novel antileukemic drugs.

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Abbreviations used: ALL, acute lymphoblastic leukemia; B-ALL, B cell precursor ALL; CM, conditional medium; DFS, disease-free survival; DHPLC, denaturing HPLC; ERK, extracellular signal-regulated kinase; OS, overall survival; T-ALL, T cell ALL.

Acute lymphoblastic leukemia (ALL) comprises a biologically heterogeneous group of clonal disorders that originate from the uncontrolled proliferation and expansion of immature lymphoblastic cells and are characterized by an extremely variable clinical outcome (1, 2). In recent years, substantial progress has been made toward understanding the molecular events contributing to malignant transformation. This has permitted the recognition of relevant prognostic factors and risk stratification, and has favored the implementation of therapeutic approaches based on cytogenetic and molecular lesions (2–5). Despite

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these accomplishments, long-term survival in adults with ALL remains largely unsatisfactory, making the design of novel antileukemic drugs tailored to specific biological targets a current priority (4, 6).

The four mammalian members of the JAK family (JAK1, JAK2, JAK3, and TYK2) are nonreceptor tyrosine kinases functioning as signal transducers to control cellular proliferation, survival, and differentiation (7, 8). JAK proteins associate constitutively with a variety of cytokine receptors lacking intrinsic kinase activity, and promote signal flow by phosphorylating tyrosyl residues of activated receptors to allow the recruitment and activation of STAT proteins. They share a complex multidomain structure characterized by a tyrosine kinase domain at the C terminus, which is flanked by a catalytically inactive pseudokinase domain with regulatory function. Their N-terminal half contains a FERM homology domain, which is implicated in receptor binding and possibly regulates the catalytic activity of the kinase, and an adjacent SH2-like domain. In contrast to their conserved structure, increasing experimental data indicate that JAK family members preferentially associate with a diverse subset of cytokine receptors, each differentially expressed by individual cell lineages and tissues, facilitating specificity in function (9). Among them, JAK1 plays an essential and nonredundant role in mediating biological responses induced by a specific subgroup of cytokines controlling lymphoid cell precursor development (10). *Jak1*$^{-/-}$ mouse pups exhibit a thymus that is markedly reduced in size, which is associated with a severe decrease in cellularity. Jak1 loss of function is also associated with profound abnormalities in the B cell compartment caused by a block in differentiation at the pro–B/pre–B cell transition step, resulting in a deficit in the production of mature B lymphocytes (10). Based on its crucial role in lymphocyte proliferation, survival, and differentiation, we hypothesized that up-regulation of JAK1 signaling might contribute to malignancies of the lymphoid lineage. In this study, we report that somatic activating *JAK1* mutations occur among adults with T cell precursors ALL, and are associated with poor response to therapy and overall prognosis.

RESULTS AND DISCUSSION

JAK1 mutation analysis in ALL

To explore possible contributions of somatic *JAK1* gene mutations in ALL, genomic DNA samples from BM aspirates of adult subjects with B cell precursor ALL (B-ALL; $n = 88$) and T cell ALL (T-ALL; $n = 38$) obtained at diagnosis and before therapy were screened for mutations in the entire *JAK1* coding region using denaturing HPLC (DHPLC). Direct sequencing of variant elution profiles allowed the identification of 37 intronic and exonic changes, including 9 nonsynonymous variants observed in 14 individuals (Table I and Table S1, available at http://www.jem.org/cgi/content/full/jem.20072182/DC1). Among the missense defects, genotyping of genomic DNAs from BM obtained during remission demonstrated the somatic origin of the 1535C>T (Ser512Leu), 1901C>A (Ala634Asp), and 2171G>A (Arg724His) changes in the leukemic clones (Fig. 1 A and Table I). To verify that the nonsynonymous substitutions identified in patients for whom nonleukemic DNA was not available were not gene variants occurring in the population, 335 population-matching control individuals were analyzed, and none harbored the 611A>T (Lys204Met), 2635C>A (Arg879Ser), 2635C>T (Arg879Cys), and 2636G>A (Arg879His) changes or other defects altering those codons. Although the T-ALL–restricted occurrence of three distinct substitutions affecting Arg^{879} (3/38 versus 0/335; Fisher's exact probability < 0.001) further supported the relevance of the substitution of this residue, we could not exclude that the 611A>T change might represent a private neutral variant. The two remaining missense changes, 184A>G (Ile62Val) and 1078C>T (Arg360Trp), were deemed nonpathogenic variants, as they were observed in nonleukemic cells of affected patients or in unaffected control subjects.

All mutations occurred as heterozygous changes and affected conserved residues within the FERM, SH2, pseudokinase, and kinase domains (Fig. 1 B). In two cases, DHPLC profiles and electropherograms indicated that the mutant allele might be present in only a fraction of leukemic cells, suggesting that these lesions did not represent early events during leukemogenesis but were acquired during disease progression (Fig. 1 C). Remarkably, mutations were relatively common among individuals with T-ALL (18.4% of cases, 95% CI = 7.7–34.3%), whereas they occurred in only three patients with B-ALL (3.4% of cases, 95% CI = 0.7–9.6%; Table I). Such a difference in mutation prevalence between cohorts was statistically significant (Fisher's exact probability = 0.003). DHPLC screening performed on affected exons by using pooled DNAs excluded loss of the normal allele and a homozygous condition for a gene variant caused by mitotic recombination in all cases.

To investigate the prevalence of *JAK1* mutations among pediatric ALL cases, genomic DNA from BM obtained at diagnosis was scanned for mutations in affected exons. No lesion was observed within the B-ALL cohort ($n = 85$), whereas a nonsynonymous 1957C>T transition (Leu653Phe) was identified in 1 of 49 subjects with T-ALL (2.0% of cases, 95% CI = 0.05–10.9%). This mutation was not observed in the BM obtained from the patient at the time of remission, indicating that it was somatically acquired in the leukemic cells.

Overall, these results indicated that *JAK1* gene mutations occur in ALL and are more frequently observed among adult individuals with involvement of the T cell lineage.

Functional consequences of somatic *JAK1* mutations

To examine the effects of the identified mutations on protein function, WT JAK1 or a mutant form (A634D, R724H, and R879C) was expressed transiently in JAK1-defective human fibrosarcoma U4C cells, and endogenous STAT1 phosphorylation was compared basally and after stimulation with IFN-γ (Fig. 2 A). Consistent with previous studies (10, 11), untransfected cells lacking functional JAK1 did not exhibit STAT1 phosphorylation in response to IFN-γ. All JAK1 mutants promoted an enhanced response to the ligand compared with

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Table I. List of nonsynonymous *JAK1* changes identified in subjects with ALL

Cohort and lineage	Number of cases	Nucleotide substitution	Exon	Amino acid substitution	Domain	Mutation/ polymorphism
Adult ALL						
B-ALL	88					
	1	184A>G	2	Ile62Val	FERM	Polymorphism[a]
	1	611A>T	5	Lys204Met	FERM	Mutation[b]
	1	1901C>A	13	Ala634Asp	Pseudokinase	Mutation
	1	2171G>A	15	Arg724His	Pseudokinase	Mutation[d]
T-ALL	38					
	1	184A>G	2	Ile62Val	FERM	Polymorphism[a]
	1	1078C>T	7	Arg360Trp	FERM	Polymorphism[c]
	1	1535C>T	10	Ser512Leu	SH2	Mutation[d]
	1	1901C>A	13	Ala634Asp	Pseudokinase	Mutation[d]
	3	2171G>A	15	Arg724His	Pseudokinase	Mutation[e]
	1	2635C>A	18	Arg879Ser	Kinase	Mutation[b,f]
	1	2635C>T	18	Arg879Cys	Kinase	Mutation[b]
	1	2636G>A	18	Arg879His	Kinase	Mutation[b]
Childhood ALL						
B-ALL	85					
T-ALL	49					
	1	1957C>T	13	Leu653Phe	Pseudokinase	Mutation[d]

[a]This change was observed in unaffected individuals.
[b]This change was not present among 335 population-matching control individuals.
[c]This change was present at remission.
[d]This change was not present at remission.
[e]This change was not present at remission in the one individual analyzed.
[f]This subject carried a concomitant 2171G→A change.

WT JAK1. Of note, basal STAT1 phosphorylation was observed in cells expressing the A634D mutant, suggesting ligand-independent up-regulation of the kinase. Consistent with this, expression of the A634D mutant resulted in an essentially constitutive STAT1 transcriptional activation, whereas a statistically significant increase in STAT1 activity was observed in U4C cells expressing both the R724H and R879C mutants, basally and after stimulation (Fig. 2 B).

To further assess the ability of mutations to up-regulate signal flow, we transduced Ba/F3 cells with WT *Jak1* or each of the selected mutants to evaluate whether their expression induced autonomous growth of cytokine-dependent cells. GFP-expressing Ba/F3 cells were selected by flow cytometry, cultured in 5 or 0.5% WEHI-3B cell conditional medium (CM) as a source of IL-3, as well as in absence of the cytokine, and counted to assess proliferation (Fig. 2 C). Three independent experiments indicated that expression of the A634D and, with less efficiency, R724H Jak1 mutants conferred IL-3–independent growth to cells, whereas cells expressing WT Jak1 or the R879C Jak1 mutant retained dependence on the cytokine for survival. Of note, cells expressing each of the three mutants exhibited enhanced growth in response to IL-3. Consistent with these findings, Ba/F3 cells expressing the A634D Jak1 mutant exhibited enhanced Stat5, Akt, and extracellular signal-regulated kinase (ERK) phosphorylation basally and after stimulation, whereas a higher phosphorylation level of these signal transducers in cells expressing the R724H Jak1 protein was observed in cultures maintained in the presence of IL-3 (Fig. 2 D). Notably, we did not observe Jak1 phosphorylation in parental and transduced Ba/F3 cells basally and cultured in 2% WEHI-3B cell CM; however, phosphorylation was appreciable in A634D and R724H Jak1-transduced cells after selection (7-d culture in absence of IL-3; unpublished data).

IL-9 protects the T cell lymphoma BW5147 cell line against dexamethasone-induced apoptosis (12), an effect that is dependent on Jak1-mediated Stat3 and Stat5 activation (13). To demonstrate the activating role of the ALL-associated *JAK1* mutations in a different cellular system, BW5147 cells were transduced with WT *Jak1* or each of the selected mutants to evaluate their effect on this stress response. GFP-expressing cells were isolated by flow cytometry, cultured in the presence of cyclosporine A and dexamethasone, with or without IL-9, and [^{3}H]thymidine incorporation was determined to assess proliferation (Fig. 2 E). Three independent experiments documented that expression of the A634D and R879C Jak1 mutants, but not R724H Jak1, conferred increased growth to cells basally. Of note, whereas transduced cells exhibited comparable responses to high levels of IL-9, those expressing the A634D and R879C Jak1 mutants were more responsive to low levels of the cytokine. Expression of the A634D Jak1, but not that of the R879C mutant, was associated with enhanced phosphorylation of Jak1, Stat3, and Stat5 basally (unpublished data).

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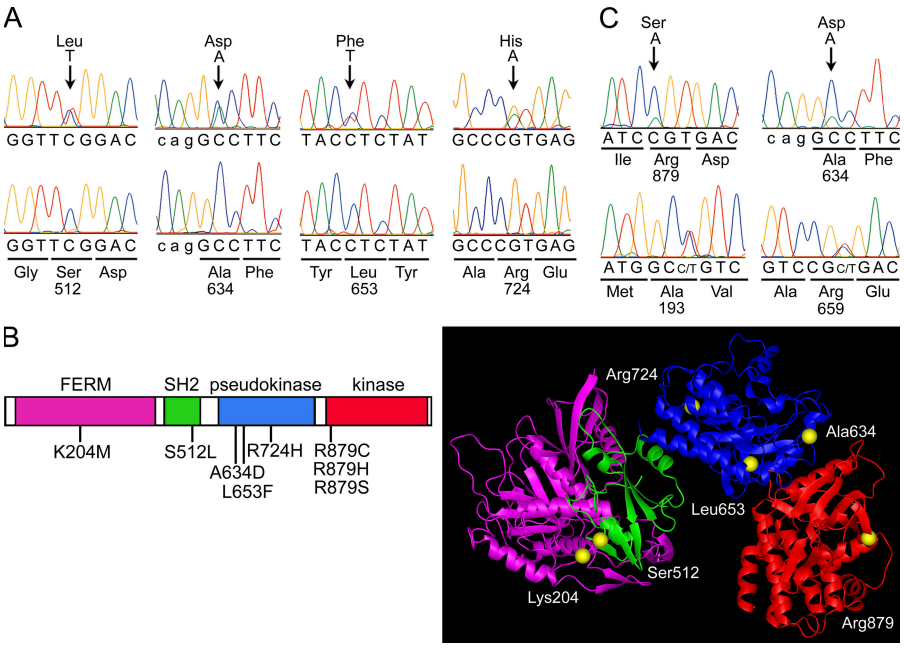

Figure 1. Somatic *JAK1* mutations in ALL. (A) Representative electropherograms showing the occurrence of somatically acquired *JAK1* mutations in subjects with T-ALL. In all cases, mutations were observed at diagnosis (top), but were undetectable during remission (bottom). (B) JAK1 domain structure and location of affected residues. The predicted amino acid substitutions resulting from the *JAK1* mutations are positioned below the diagram of the protein with its functional domains indicated (left) and shown in JAK1 three-dimensional modeled structure (right). (C) Electropherograms showing the occurrence of mutations in a fraction of leukemic cells of two individuals with T-ALL. In both patients, the mutant allele constituted only a portion of the amplified fragment from BM obtained at diagnosis (blasts >70% of total cells; top). The heterozygous status of each subject for an intragenic polymorphic site (bottom) is shown for comparison.

Overall, these data indicated that the three selected leukemia-associated *JAK1* mutants are hypermorphs, with A634D Jak1 having a seemingly stronger effect, and that different mechanisms are likely to be involved in their cell context–related gain of function.

Molecular modeling of JAK1 and location of affected residues

To look at the structural causes resulting in JAK1 functional up-regulation, we generated a model of JAK1 structure because no crystallographic information was available for this protein. Energy-minimized models of each of the four domains were generated separately by homology to available crystallographic structures of proteins with similar sequences and overall fold. The quaternary arrangement of the four domains was then determined by superimposing the models on a predicted three-dimensional structure of JAK2 (14). According to the superimposed structure, Ala^{634} and Leu^{653} are placed on the surface of the pseudokinase domain involved in the interaction with the kinase domain (Fig. 1 B). Based on the evidence supporting a negative regulatory role of the pseudokinase domain on catalytic function of JAK proteins (15–17), the pathogenetic mechanism of the A634D and L653F changes is predicted to involve a looser interdomain interaction, relaxing inhibitory control on the kinase activity. Consistent with this hypothesis, substitution of two residues located in the pseudokinase domain at the interface with the kinase domain in JAK2 (Val^{617}) and JAK3 (Ala^{572}) promote increased catalytic activity basally (Fig. S1, available at http://www.jem.org/cgi/content/full/jem.20072182/DC1) (18–20). Our model also predicts that residues Lys^{204} and Ser^{512} would perturb the SH2–FERM interdomain interaction because they are located at the interface between these domains, approximately facing each other. This finding is noteworthy because it has been proposed that JAK1's SH2 domain does not function as a phosphotyrosyl-binding domain, but instead plays a structural role in stabilizing the conformation of the FERM domain (21), which mediates its association to cytokine receptors and exerts an as yet uncharacterized restraint on catalytic function (22, 23). The molecular mechanism through which these mutations affect JAK1 function remains to be explained. Structural and functional consequences were not obvious for the activating changes affecting residues Arg^{724} and Arg^{879}.

Gene expression profile analysis in blasts with a mutated *JAK1* allele

Total RNA was available from blasts of 5 *JAK1* mutation-positive (S512L, A634D, and R724H) and 11 mutation-negative subjects of the T-ALL cohort. Unsupervised clustering based on 1,345 probe sets selected by nonspecific filtering clustered expression profiles of mutation-positive blasts into two clusters, suggesting contribution of *JAK1* mutations to

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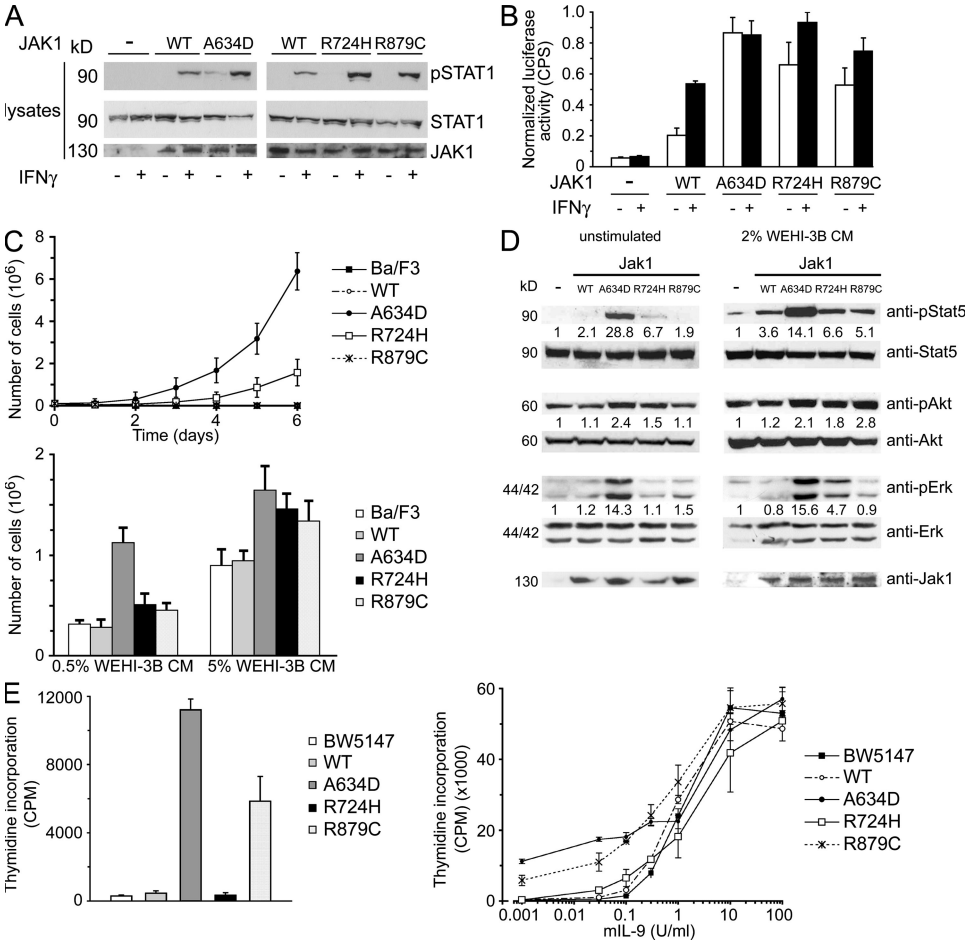

Figure 2. Functional effects of leukemia-associated *JAK1* mutations. (A) STAT1 phosphorylation assays. Basal and IFN-γ–stimulated endogenous STAT1 phosphorylation in JAK1-defective U4C cells transiently transfected with WT *JAK1* or selected mutants. Blots are representative of three experiments. (B) STAT1 activation assays. Basal and IFN-γ–stimulated endogenous STAT1 transcriptional activity in JAK1-defective U4C cells transiently cotransfected with p-GAS-Luc and phRL-TK constructs, and WT *JAK1* or a mutant allele. STAT1-induced luciferase gene expression levels were determined by measuring the luciferase activity (CPS, counts per second) normalized to the activity of the Renilla luciferase, using a dual-luciferase reporter assay system. Activity ratios are expressed as the mean of three replicates ± the SD. (C) Ba/F3 survival assays. WT or mutant *Jak1*-transduced Ba/F3 cells were grown in the absence of IL-3 (top) or with 0.5 or 5% WEHI-3B cell CM as source of IL-3 (bottom). Cell numbers (mean of three replicates ± the SD) were counted at the indicated time points (top) or at day 3 of culture (bottom). (D) Stat5, Akt, and ERK phosphorylation assays. Endogenous Stat5 Tyr694, Akt Ser473, and ERK1/2 Thr202/Tyr204 phosphorylation levels from lysates of Ba/F3 cells transduced with WT Jak1 or a mutant form and cultured without IL-3 (left) or with 2% WEHI-3B cell CM as the source of IL-3 (right). Activation of Stat5 (pStat5/Stat5), AKT (pAkt/Akt), and ERK1/2 (pERK/ERK) is expressed as a multiple of activation in untransduced cells. Blots are representative of at least three experiments performed. (E) BW5147 proliferation assays. WT or mutant *Jak1* transduced BW5147 cells were grown in presence of 200 µg/ml dexamethasone and 50 µg/ml cyclosporine A, in absence (left) or with different concentrations (right) of IL-9. After 72 h, proliferation was measured after [^{3}H]thymidine was added to the cultures (6 h). Data are shown as the means of three replicates ± the SD. For convenience, the amino acid changes affecting residues Ala^{633}, Arg^{723}, and Arg^{878} of the murine Jak1 protein (encoded by the pMX-Jak1-IRES-GFP constructs used to transduce the Ba/F3 and BW5147 cell lines) are indicated according to the homologous residues in human JAK1.

distinct major mechanisms of deregulation (Fig. S2, available at http://www.jem.org/cgi/content/full/jem.20072182/DC1). Notably, supervised gene expression analysis revealed a distinctive expression signature shared by leukemic cells with a mutated *JAK1* gene (Fig. 3 A) based on the expression of 133 differentially expressed probe sets, consisting of 112 differentially expressed genes, the majority of them being overexpressed in *JAK1* mutation-positive samples (Table S2). Among the up-regulated genes, those whose transcription was known to be positively modulated by JAK/STAT signaling, including *IRF1*, *SOCS3*, *ISG15*, *ISGF3G*, *IFI44L*, and *IRF7*, were overrepresented in all the JAK1 mutation-positive subjects, further supporting the gain-of-function role of the ALL-associated *JAK1* lesions.

Clinical relevance of somatic *JAK1* mutations

The clinical relevance of *JAK1* mutations within the adult T-ALL cohort was investigated. Although no statistically

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significant difference was observed in white blood cell counts, gender distribution, or association with specific chromosomal rearrangements, patients with a mutated *JAK1* allele tended to have a more advanced age at diagnosis (median = 40.6 vs. 24.2; P < 0.01), which was consistent with the lower prevalence of mutations identified among children and adolescents with T-ALL included in the study. Comparison of the response to therapy between *JAK1* mutation-positive and -negative patients indicated a higher percentage of cases exhibiting resistance to induction therapy in the former (43 vs. 20%), although this difference did not reach statistical significance caused by the relatively small size of the study cohort. Consistent with that finding, a statistically significant reduced disease-free survival (DFS; median =8.7 vs. 20.5 mo; P = 0.01) and overall survival (OS; median = 10.6 vs. 32.5; P < 0.01) was observed among *JAK1* mutation-positive patients (Fig. 3 B). Multivariate analysis confirmed the statistical significance of these associations (DFS: HR= 6.20, 95% CI = 1.32–29.09, P = 0.02; OS: HR = 2.82, 95% CI = 1.07–7.48, P = 0.04), and excluded a significant contribution of patients' age (DFS: HR = 0.99, 95% CI = 0.93–1.05, P = 0.64; OS: HR= 1.01, 95% CI = 0.97–1.05, P = 0.60).

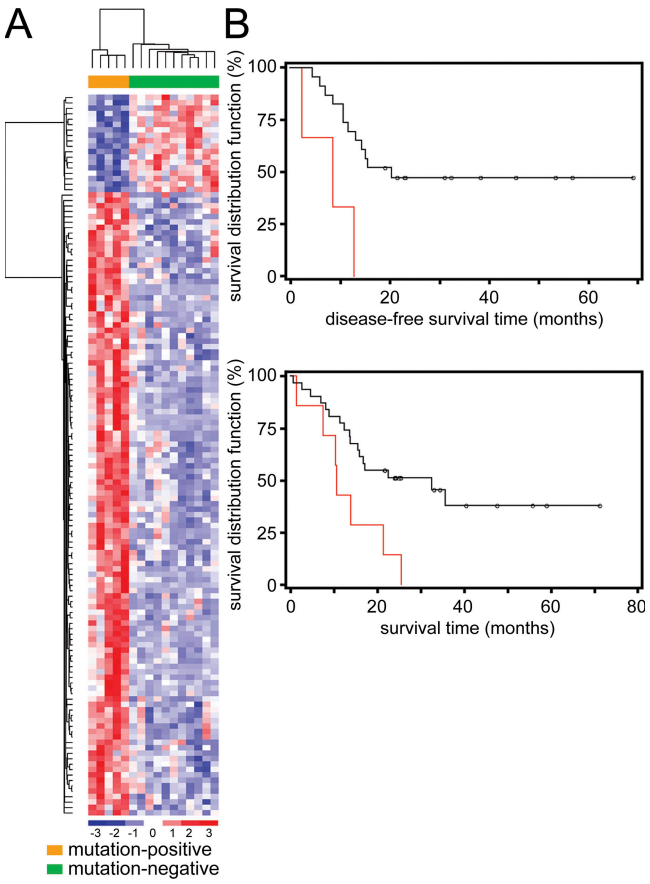

Figure 3. Gene expression profiles and clinical relevance of somatic *JAK1* mutations in adult T-ALL. (A) Supervised hierarchical clustering of gene expression profiles performed on blasts from 16 adult T-ALL patients, with (orange) or without (green) a *JAK1* mutation. (B) Kaplan-Meier estimates of DFS (top) and OS (bottom) in subjects with (red) or without (black) a *JAK* mutation. Multivariate analysis confirmed the statistical significance of the reduced DFS and OV among *JAK1* mutation-positive patients, and excluded a significant contribution of the more advanced age of these subjects.

In the adult T-ALL cohort, the mutation status for *NRAS*, *KRAS*, *NOTCH1*, and *PTEN* was also assessed (unpublished data). Among the *JAK1* mutation-positive cases, no defect was observed in the *NRAS*, *KRAS*, and *PTEN* genes, although such mutations had a relatively low prevalence in the entire adult T-ALL cohort (*RAS* genes: 11% of cases, 95% CI = 2.9–24.8%; *PTEN*: 14% of cases, 95% CI = 4.5–28.8%). In contrast, heterozygous mutations in *NOTCH1* were observed in all *JAK1* mutation-positive individuals. Given the high prevalence of *NOTCH1* defects observed in the cohort (70% of cases, 95% CI = 53.0–84.1%), this association was not statistically significant (P = 0.06). This observation, however, suggests that activation of *JAK1* and *NOTCH1* transduction pathways might cooperate in T-ALL pathogenesis and/or progression. No significant difference in response to therapy or outcome was observed between *NOTCH1* mutation-positive and -negative patients. Interestingly, *NOTCH1* and *PTEN* mutations exhibited a mutually exclusive distribution because none of the five subjects carrying *PTEN* lesions had a *NOTCH1* defect (Fisher's exact probability = 0.001).

Aberrant *JAK1* function and leukemogenesis

Functional up-regulation of two members of the JAK family, JAK2 and JAK3, has recently been discovered in myeloproliferative disorders and other malignancies of the myeloid lineage (18–20, 24). The JAK2 V617F amino acid change occurs in the majority of polycythemia vera cases and in ~50% of individuals with essential thrombocythemia or idiopathic myelofibrosis. The available data support the view that this recurrent change, which affects the pseudokinase domain of the protein, induces constitutive activation of the kinase and hypersensitivity to cytokines. Similarly, three *JAK3* hypermorphic alleles promoting cytokine independence in Ba/F3 cells have been identified in acute megakaryoblastic myeloid leukemia. In this study, we showed that somatically acquired activating *JAK1* mutations occur in ALL, particularly in adults, further emphasizing the importance of JAK-mediated signaling dysregulation in leukemogenesis and extending the spectrum of hematologic malignancies associated with aberrant activation of this signal transduction pathway. Even though the molecular mechanisms by which individual *JAK1* mutations promote gain of function are likely to be diverse and remain to be fully characterized, modeling and biochemical data are consistent with the view that, similar to what has been observed for somatic leukemia-associated *JAK2* and *JAK3* defects, most mutations would interfere with the autoinhibitory control on the catalytic activity. For most mutations, this effect would be achieved by triggering local structural rearrangements in regions involved in interdomain interactions between the

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pseudokinase and kinase domains or the FERM and SH2 domains (Fig. S1).

JAK1 is expressed widely and participates in intracellular signaling elicited by class II cytokine receptors and receptors that use the gp130 or γ_c receptor subunit. Although the hematopoietic defects in *Jak1*$^{-/-}$ mice were restricted to the lymphoid cell compartment as a result of an impaired response to IL-7 (10) and the present findings indicate a cell-context dependence of somatically acquired *JAK1* mutations' contribution to leukemogenesis, we cannot exclude the involvement of this kinase in other malignancies. We speculate that a concomitant genetic event, including a mutation affecting other members of the JAK family, might synergize with the *JAK1* defect to promote aberrant cell proliferation and/or survival in a cell-specific context. This hypothesis is currently under investigation.

Although 70–80% of pediatric patients with either B- or T-ALL have excellent long-term response to intensive combination chemotherapy, adult patients exhibit a less favorable outcome (4, 25). In B- ALL, such a poor prognosis has been associated in part with the presence of *BCR/ABL* or *ALL1/AF4* gene rearrangements. In contrast, the unfavorable outcome of adult patients with T-ALL has not conclusively been attributed to any cytogenetic lesion, albeit the prognostic relevance of aberrant *ERG* and *TLX1* gene expression and *NOTCH1* mutations has recently been reported (26–28). The present work provides the first evidence that *JAK1* gene defects are associated with a poor response to therapy, frequent relapse of the disease, and reduced OS, identifying such mutations as a novel informative prognostic marker occurring in a sizable proportion of adult T-ALL. Although studies on larger cohorts are required to determine more precisely the clinical relevance and prognostic value of *JAK1* defects in adult and pediatric ALL, our findings provide a rationale for the development of novel therapeutic approaches tailored at interfering with JAK1 signaling, encouraging studies aimed at testing the efficacy and side effects of JAK1 inhibitors in the management of adult T-ALL patients.

MATERIALS AND METHODS

All cohorts and molecular analyses. Cohorts studied included patients enrolled in the Gruppo Italiano Malattie Ematologiche dell'Adulto 0496 and 2000 (adults), and Associazione italiana ematologia ed oncologia pediatrica ALL 2000 (children and adolescents) clinical trials. Written informed consent for genetic analyses was obtained from all subjects according to the Declaration of Helsinki. BM mononuclear cells were isolated by density gradient centrifugation, and then cryopreserved. Genomic DNA was isolated in a standard fashion. Exons 26, 27, and 34 of the *NOTCH1* gene were analyzed by direct sequencing, whereas the entire *JAK1* and *PTEN* coding regions and exons 1 and 2 of *NRAS* and *KRAS* were screened by DHPLC (Wave 2100 System; Transgenomic) and sequencing. Primer sequences are available upon request. Gene expression profiling methodology is described in the Supplemental materials and methods (available at http://www.jem.org/cgi/content/full/jem.20072182/DC1).

Functional studies. The mutations resulting in the A634D, R724H, and R879C changes were introduced by site-directed mutagenesis in a VSV-tagged *JAK1* cDNA cloned in pRc/CMV vector provided by S. Pellegrini (Pasteur Institute, Paris, France). JAK1-defective human fibrosarcoma U4C cells (provided by S. Pellegrini) were maintained in DME supplemented with 10% heat-inactivated FCS and antibiotics. To evaluate endogenous STAT1 phosphorylation, after starvation (24 h), transiently transfected cells were stimulated with IFN-γ (1,000 U/ml, 15 min) or left unstimulated. Lysed samples were analyzed by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane (Pierce Chemical Co.) and probed with anti–phospho-Stat1 (9171; Cell Signaling Technology), anti-Stat1 (9172; Cell Signaling Technology), and anti-Jak1 (3332; Cell Signaling Technology) antibodies. Endogenous STAT1 transcriptional activity was assessed by luciferase transactivation assays in cells cotransfected with a p-GAS-Luc construct, switched to serum-starvation medium (8 h), and then stimulated with IFN-γ (1,000 U/ml, 16 h) or left unstimulated. STAT1-induced luciferase expression was assessed and normalized using a dual luciferase reporter assay system (Promega) and a phRL-TK plasmid constitutively expressing the Renilla luciferase.

Each of the three leukemia-associated mutations was also introduced in the murine *Jak1* cDNA cloned in the bicistronic retroviral vector pMX-IRES-GFP. Constructs were transfected into Phoenix or BOSC packaging cells to produce retroviruses, and murine Ba/F3 (maintained in RPMI 1640 medium containing 10% FCS, 1% L-glutamine, and 10% WEHI-3B cell CM) and BW5147 (maintained in Iscove-Dulbecco's medium supplemented with 10% FBS, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine) cells were infected with retroviral supernatants. GFP-positive populations were purified by flow-cytometric sorting, and then expanded. Equal GFP expression levels of transduced cells were confirmed by FACS analysis (Fig. S3, available at http://www.jem.org/cgi/content/full/jem.20072182/DC1). Transduced Ba/F3 cells were cultured in the absence or presence of IL-3 (0.5 or 5% WEHI-3B cell CM) for assaying cytokine independence and response, and viable cells were counted by Trypan blue exclusion. Proliferation of transduced BW5147 cells cultured in the presence of 50 μg/ml cyclosporine A and 200 μg/ml dexamethasone, in the presence or absence of mIL-9, was determined using [^{3}H]thymidine incorporation assay. In brief, 3,000 cells were seeded in 96-well plates (200 μl volume medium), and after 72 h, [^{3}H]thymidine was added to the cultures for 6 h. Cells were then collected on microfilter plates, and thymidine incorporation was measured after addition of 25 μl of liquid scintillant. For Western blot studies, Ba/F3 cells were starved in RPMI 1640 medium containing 1% BSA (5 h), and then stimulated with 2% WEHI-3B cell CM (30 min) or left unstimulated, whereas BW5417 cells were starved in Iscove-Dulbecco's medium (12 h) and left unstimulated or stimulated with 100 U/ml mIL-9 for 15 min. Evaluation of Jak1, Akt, Stat3, Stat5, and ERK1/2 expression and phosphorylation levels was performed using anti–phospho-Jak1, anti-Jak1, anti–phospho-Akt, anti-Akt, anti–phospho-Stat3, anti-Stat3, anti–phospho-Stat5, anti-Stat5, anti–phospho-p44/42 ERK, and anti-p44/42 ERK antibodies (all from Cell Signaling Technology).

Structural models of the kinase, pseudokinase, and SH2 and FERM domains of JAK1 were generated as described in the online supplemental information section.

Statistical analysis. Confidence intervals of proportions (at 95% level) were calculated based on the binomial distribution. The probabilities of OS and DFS were estimated using the Kaplan-Meier method. The log-rank test was used to compare treatment effect and risk factor categories. All tests were two-sided, accepting $P \leq 0.05$ as indicating a statistically significant difference. Cox proportional hazard models, including age and *JAK1* as variables, were used to perform multivariate analyses for OS and DFS. The SAS software (SAS Institute) was used for the analysis.

Online supplemental material. Methodologies and approaches used to generate the JAK1 molecular modeling and to perform and analyze adult T-ALL leukemic cell gene expression profiles (including cited references) are provided in the Supplemental Materials and methods. Table S1 is a list of the

intronic or synonymous *JAK1* changes identified in the study. Table S2 is a list of differentially expressed genes in *JAK1* mutation-positive versus mutation-negative adult subjects with T-ALL. Fig. S1 shows the JAK1 domain structure and the location of JAK1, JAK2, and JAK3 residues reported to be mutated in myeloproliferative disorders and leukemias. Fig. S2 shows the unsupervised hierarchical clustering of gene expression profiles of blasts from 16 adult T-ALL patients, with or without a *JAK1* mutation. Fig. S3 shows the GFP expression levels of purified Ba/F3 and BW5417 cells transduced with a bicistronic retroviral vector pMX-Jak1-IRES-GFP coding for WT Jak1 or one of the three generated mutants. The online version of this article is available at http://www.jem.org/cgi/content/full/jem.20072182/DC1.

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REFERENCES

1. Armstrong, S.A., and A.T. Look. 2005. Molecular genetics of acute lymphoblastic leukemia. *J. Clin. Oncol.* 23:6306–6315.
2. Pui, C.H., and W.E. Evans. 2006. Treatment of acute lymphoblastic leukemia. *N. Engl. J. Med.* 354:166–178.
3. Yeoh, E.J., M.E. Ross, S.A. Shurtleff, W.K. Williams, D. Patel, R. Mahfouz, F.G. Behm, S.C. Raimondi, M.V. Relling, A. Patel, et al. 2002. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell.* 1:133–143.
4. Vitale, A., A. Guarini, S. Chiaretti, and R. Foa. 2006. The changing scene of adult acute lymphoblastic leukemia. *Curr. Opin. Oncol.* 18:652–659.
5. Chiaretti, S., X. Li, R. Gentleman, A. Vitale, M. Vignetti, F. Mandelli, J. Ritz, and R. Foa. 2004. Gene expression profile of adult T-cell acute lymphocytic leukemia identifies distinct subsets of patients with different response to therapy and survival. *Blood.* 103:2771–2778.
6. Thomas, X. 2005. Emerging drugs for adult acute lymphoblastic leukaemia. *Expert Opin. Emerg. Drugs.* 10:591–617.
7. Yamaoka, K., P. Saharinen, M. Pesu, V.E. Holt III, O. Silvennoinen, and J.J. O'Shea. 2004. The Janus kinases (Jaks). *Genome Biol.* 5:253.
8. Schindler, C., D.E. Levy, and T. Decker. 2007. JAK-STAT signaling: from interferons to cytokines. *J. Biol. Chem.* 282:20059–20063.
9. Murray, P.J. 2007. The JAK-STAT signalling pathway: Input and output integration. *J. Immunol.* 178:2623–2629.
10. Rodig, S.J., M.A. Meraz, J.M. White, P.A. Lampe, J.K. Riley, C.D. Arthur, K.L. King, K.C. Sheehan, L. Yin, D. Pennica, et al. 1998. Disruption of the Jak1 gene demonstrates obligatory and nonredundant roles of the Jaks in cytokine-induced biologic responses. *Cell.* 93:373–383.
11. Müller, M., J. Briscoe, C. Laxton, D. Guschin, A. Ziemiecki, O. Silvennoinen, A. Harpur, G. Barbieri, B.A. Witthuhn, C. Schindler, S. Pellegrini, A.F. Wilks, J.N. Ihle, G.R. Stark, and L.M. Kerr. 1993. The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. *Nature.* 366:129–135.
12. Renauld, J.C., A. Vink, J. Louahed, and J. Van Snick. 1995. Interleukin-9 is a major anti-apoptotic factor for thymic lymphomas. *Blood.* 85:1300–1305.
13. Demoulin, J.B., C. Uyttenhove, E. Van Roost, B. DeLestre, D. Donckers, J. Van Snick, and J.C. Renauld. 1996. A single tyrosine of the interleukin-9 (IL-9) receptor is required for STAT activation, antiapoptotic activity, and growth regulation by IL-9. *Mol. Cell. Biol.* 16:4710–4716.
14. Giordanetto, F., and R.T. Kroemer. 2002. Prediction of the structure of human Janus kinase 2 (JAK2) comprising JAK homology domains 1 though 7. *Protein Eng.* 15:727–737.
15. Chen, M., A. Cheng, F. Candotti, Y.J. Zhou, A. Hymel, A. Fasth, L.D. Notarangelo, and J.J. O'Shea. 2000. Complex effects of naturally occurring mutations in the JAK3 pseudokinase domain: evidence for interactions between the kinase and pseudokinase domains. *Mol. Cell. Biol.* 20:947–956.
16. Lindauer, K., T. Loerting, K.R. Liedl, and R.T. Kroemer. 2001. Prediction of the structure of human Janus kinase 2 (JAK2) comprising the two carboxy-terminal domains reveals a mechanism for autoregulation. *Protein Eng.* 14:27–37.
17. Saharinen, P., and O. Silvennoinen. 2002. The pseudokinase domain is required for suppression of basal activity of Jak2 and Jak3 tyrosine kinases and for cytokine-inducible activation of signal transduction. *J. Biol. Chem.* 277:47954–47963.
18. James, C., V. Ugo, J.P. Le Couedic, J. Staerk, F. Delhommeau, C. Lacout, L. Garcon, H. Raslova, R. Berger, A. Bennaceur-Griscelli, et al. 2005. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature.* 434:1144–1148.
19. Levine, R.L., M. Wadleigh, J. Cools, B.L. Ebert, G. Wernig, B.J. Huntly, T.J. Boggon, I. Wlodarska, J.J. Clark, S. Moore, et al. 2005. Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. *Cancer Cell.* 7:387–397.
20. Walters, D.K., T. Mercher, T.L. Gu, T. O'Hare, J.W. Tyner, M. Loriaux, V.L. Goss, K.A. Lee, C.A. Eide, M.J. Wong, et al. 2006. Activating alleles of JAK3 in acute megakaryoblastic leukemia. *Cancer Cell.* 10:65–75.
21. Radtke, S., S. Haan, A. Jorissen, H.M. Hermanns, S. Diefenbach, T. Smyczek, H. Schmitz-Vandeleur, P.C. Heinrich, I. Behrmann, and C. Haan. 2005. The Jak1 SH2 domain does not fulfill a classical SH2 function in Jak/STAT signaling but plays a structural role for receptor interaction and up-regulation of receptor surface expression. *J. Biol. Chem.* 280:25760–25768.
22. Haan, C., H. Is'harc, H.M. Hermanns, H. Schmitz-Van De Leur, I.M. Kerr, P.C. Heinrich, J. Grotzinger, and I. Behrmann. 2001. Mapping of a region within the N terminus of Jak1 involved in cytokine receptor interaction. *J. Biol. Chem.* 276:37451–37458.
23. Zhou, Y.J., M. Chen, N.A. Cusack, L.H. Kimmel, K.S. Magnuson, J.G. Boyd, W. Lin, J.L. Roberts, A. Lengi, R.H. Buckley, et al. 2001. Unexpected effects of FERM domain mutations on catalytic activity of Jak3: structural implication for Janus kinases. *Mol. Cell.* 8:959–969.
24. Levine, R.L., M. Loriaux, B.J. Huntly, M.L. Loh, M. Beran, E. Stoffregen, R. Berger, J.J. Clark, S.G. Willis, K.T. Nguyen, et al. 2005. The JAK2V617F activating mutation occurs in chronic myelomonocytic leukemia and acute myeloid leukemia, but not in acute lymphoblastic leukemia or chronic lymphocytic leukemia. *Blood.* 106:3377–3379.
25. Gokbuget, N., and D. Hoelzer. 2006. Treatment of adult acute lymphoblastic leukemia. *Hematology (Am Soc Hematol Educ Program).* 133–141.
26. Baldus, C.D., T. Burmeister, P. Martus, S. Schwartz, N. Gokbuget, C.D. Bloomfield, D. Hoelzer, E. Thiel, and W.K. Hofmann. 2006. High expression of the ETS transcription factor ERG predicts adverse outcome in acute T-lymphoblastic leukemia in adults. *J. Clin. Oncol.* 24:4714–4720.
27. Ferrando, A.A., D.S. Neuberg, R.K. Dodge, E. Paietta, R.A. Larson, P.H. Wiernik, J.M. Rowe, M.A. Caligiuri, C.D. Bloomfield, and A.T. Look. 2004. Prognostic importance of TLX1 (HOX11) oncogene expression in adults with T-cell acute lymphoblastic leukaemia. *Lancet.* 363:535–536.
28. Zhu, Y.M., W.L. Zhao, J.F. Fu, J.Y. Shi, Q. Pan, J. Hu, X.D. Gao, B. Chen, J.M. Li, S.M. Xiong, et al. 2006. NOTCH1 mutations in T-cell acute lymphoblastic leukemia: prognostic significance and implication in multifactorial leukemogenesis. *Clin. Cancer Res.* 12:3043–3049.

ALL-associated JAK1 Mutants Activate the JAK/STAT Pathway via Interleukin-9 Receptor Alpha Homodimers

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Preamble

After we (166) and others (167,168) identified activating mutations in JAK1 in ALL patients the list of oncogenic JAK mutants, the list led by the JAK2 V617F mutation associated to myeloproliferative neoplasms, steadily expanded.
We next sought to study the mechanism by which JAK1 mutants induce constitutive signaling and STAT activation. For JAK2 V617F, it was demonstrated that association with a homodimeric type I cytokine receptor such as EpoR or TpoR was necessary for constitutive signaling. Preformed dimerization of these receptors was proposed to mediate activation and signaling by JAK2 V617F (97,98). Therefore, we speculated that JAK1 mutants would also need to associate to receptors to mediate constitutive signaling.

The key observation in this paper is that JAK1 V658F and A634D fail to activate the JAK/STAT pathway upon ectopic expression in HEK293 cells, unless a JAK1-binding receptor chain such as IL-9Rα or IL-2Rβ is co-expressed. Interestingly, γc or JAK3 expression are not required for the activity of JAK1 mutants, although they are mandatory to form a ligand-responsive receptor complex. Using different experimental approaches, we show that IL-9Rα or IL-2Rβ form ligand-independent homodimers that can mediate signaling from JAK1 mutants.

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Acute Lymphoblastic Leukemia-associated JAK1 Mutants Activate the Janus Kinase/STAT Pathway via Interleukin-9 Receptor α Homodimers*[S]

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Activating mutations in JAK1 have been reported in acute lymphoblastic leukemias, but little is known about the mechanisms involved in their constitutive activation. Here, we studied the ability of JAK1 V658F and A634D to activate the Janus kinase (JAK)/STAT pathway upon ectopic expression in HEK293 cells alone or together with the other components of the interleukin-9 receptor complex (IL-9Rα, γc, and JAK3). Expression of JAK1 mutants alone failed to trigger STAT activation, but co-expression of the IL-9Rα chain promoted JAK1 mutant phosphorylation and STAT activation. Mutation of the FERM domain of JAK1, which is critical for cytokine receptor association, or of the single tyrosine of IL-9Rα involved in STAT recruitment abolished this activity, indicating that JAK1 mutants need to associate with a functional IL-9Rα to activate STAT factors. Several lines of evidence indicated that IL-9Rα homodimerization was involved in this process. IL-9Rα variants with mutations of the JAK-interacting BOX1 region not only failed to promote JAK1 activation but also acted as dominant negative forms reverting the effect of wild-type IL-9Rα. Coimmunoprecipitation experiments also showed the formation of IL-9Rα homodimers. Interestingly, STAT activation was partially inhibited by expression of γc, suggesting that overlapping residues are involved in IL-9Rα homodimerization and IL-9Rα/γc heterodimerization. Co-expression of wild-type JAK3 partially reverted the inhibition by γc, indicating that JAK3 cooperates with JAK1 mutants within the IL-9 receptor complex. Similar results were observed with IL-2Rβ. Taken together, our results show that IL-9Rα and IL-2Rβ homodimers efficiently mediate constitutive activation of ALL-associated JAK1 mutants.

Janus kinases (JAKs)[5] represent a family of four non-receptor tyrosine kinases (JAK1, JAK2, JAK3, and TYK2) that is associated with cytokine receptors of no intrinsic kinase activity (1). During the last few years several acquired JAK mutations have been identified in different malignancies. These mutations led to a gain of kinase function and are tumorigenic. The best example is the JAK2 V617F mutation associated with myeloproliferative neoplasms (2–5). JAK2 V617F retains its ability to interact with cytokine receptors (6), and an intact FERM domain, which mediates recruitment to cytokine receptors, is required for inducing transformation of hematopoietic cells (7).

At physiological levels of expression, JAK2 V617F needs to be associated to JAK2 binding homodimeric type I cytokine receptors such as the erythropoietin receptor (EPOR) or the thrombopoietin receptor (TPOR) to allow constitutive signaling (8, 9). Because EpoR is a preformed dimer in the absence of ligand (10), a model was proposed where dimerization of JAK2 V617F via interactions with a preformed EpoR dimer promotes signaling by JAK2 V617F (8). Constitutive and increased erythropoietin or thrombopoietin signaling provide a mechanism for the erythocytosis and thrombocytosis observed in these disorders (11). The A572V mutation in JAK3 has later been identified in patients with acute megakaryoblastic leukemia (12).

Recently, mutations in JAK1, such as A634D, R724H, R879C (13), and the V658F mutation (14) have been identified in adult B and T cell-acute lymphoblastic leukemia (ALL). These mutations allow for constitutive JAK1 activation when overexpressed in JAK1-deficient cell lines (11, 13), as was shown for JAK2 V617F in JAK2-deficient cell lines (2). Moreover, these A634D and R724H mutants induce the autonomous growth of the cytokine-dependent Ba/F3 cell line, whereas the A634D and R879C mutants protect the murine ALL cell line BW5147 from dexamethasone-induced apoptosis, indicating that they repre-

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[5] The abbreviations used are: JAK, Janus kinase; IL, interleukin; IL-9Rα, IL-9 receptor complex; EPOR, erythropoietin receptor; TPOR, thrombopoietin receptor; ALL, acute lymphoblastic leukemia; T-ALL, T cell ALL; STAT, signal transducers and activators of transcription; HA, hemagglutinin; FACS, fluorescence-activated cell sorter; WT, wild type.

sent gain of function mutations. However, the potential role of JAK1 binding receptors, which are all heterodimeric, in the mechanism of mutant JAK1-induced constitutive signaling has never been studied.

IL-9 is a multifunctional TH2 cytokine that was shown to be involved in T cell tumorigenesis in mouse and in humans (15–18). Moreover, *in vitro* dysregulation of the IL-9 response is associated with autonomous cell growth and malignant transformation of lymphoid cells, leading to the constitutive activation of JAK-STAT pathway (19–21). Its activities are mediated via a heterodimeric receptor complex formed by the IL-9Rα chain (IL-9Rα), which associates with JAK1, and the IL-2Rγ chain, also called γc (common γ chain), which associates with JAK3. γc is in addition involved in IL-2, -4, -7, -15, and -21 signaling, a family of cytokines involved in lymphocyte development and/or activation. IL-9Rα is sufficient to confer high affinity cytokine binding, but formation of the heterodimeric complex with γc is needed for signal transduction (21). Upon IL-9 binding, JAK1 and JAK3 are cross-activated, and IL-9Rα is phosphorylated on a single tyrosine (Tyr-116). This phosphorylated tyrosine is the only docking site for STAT1, -3, and -5, the STATS activated by IL-9 (22).

In this paper, in order to study the potential interactions between ALL-associated JAK1 mutants and the different components of IL-9 receptor complex, we co-expressed these different proteins in HEK293 cells, which lack IL-9Rα, γc, and JAK3. Our data show that JAK1 mutants alone fail to activate STAT transcriptional factors but that this process/activation is promoted by IL-9Rα homodimerization in the absence of γc and JAK3.

EXPERIMENTAL PROCEDURES

Cells and Cell Culture—HEK293 human embryonic kidney cells and COS-7 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. Human fibrosarcoma U4C cells (JAK1-deficient) and γ2A cells (JAK2-deficient) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Recombinant human IL-9 was produced in the baculovirus system in our laboratory and was purified as previously described (23). Recombinant human IL-2 was provided by Chiron.

Plasmid Constructions—V658F and A634D mouse JAK1 mutants were described previously (11, 13). Double JAK1 mutants V658F/Y107A and A634D/Y107A were generated using QuikChange XL II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The Y107A mutation was previously described to abolish the binding of JAK1 to cytokine receptors (24). Both wild-type and mutated murine JAK1 cDNAs were subcloned into the pMX-GFP biscistronic retroviral vector upstream of the internal ribosome entry site (IRES) (11, 13). Human IL-9Rα and IL-9Rα Y116F were generated as previously described (22) and were subcloned into the pREX-IRES-CD4 vector (25) or into the pEFBos-puro vector (22). The ability of murine JAK1 to transduce the signal from human IL-9Rα has been previously described (22, 26). The IL-9Rα BOX1 mutant was generated by mutation of two proline residues to two serine residues in the BOX1 motif (P*X*P to S*X*S) of wild-type IL-9Rα using the GeneEditor *in vitro* site-directed mutagenesis system (Promega, Madison, WI). Clones obtained were sequenced using DYEnamic ET Dye Terminator kit (Amersham Biosciences). The IL-9Rα IC34 construct was generated as previously described (22). Myc and HA tags were introduced after the signal sequence cleavage site in the cDNA coding for human IL-9Rα as described (10). The IL-2Rβ and IL-2Rγ (γc) were cloned to pREX-IRES-CD4 vector (25).

Dual Luciferase Assay—STAT1, STAT3, and STAT5 transcriptional activity was assessed by measurements of luciferase expression in HEK293, COS-7, U4C, and γ2A cells upon transient transfection of appropriate cDNA constructs and luciferase reporter vectors, namely pGL3-pap1-luc, pLHRE-luc, or pGRR5-luc. Another reporter plasmid, *Renilla* luciferase (pRLTk, Promega), was co-transfected as an internal transfection control. STAT5-mediated transcription was evaluated with the pLHRE-luc reporter gene constructs harboring tandem copies of the STAT5-inducible lactogenic hormone response element (LHRE) of the rat β-casein gene promoter, inserted upstream a luciferase gene (27). STAT3-mediated transcription was evaluated with pGL3-pap1-luc plasmid containing the luciferase gene under the control of the STAT3-inducible rat *Pap1* (pancreatitis-associated protein-1) promoter (28). STAT1, STAT3, and STAT5-mediated transcription was evaluated using the pGRR5-luc construct (provided by Dr. P. Brennan, Imperial Cancer Research Fund, London, UK) that contains five copies of the STAT-binding site of the FcγRI gene inserted upstream from a luciferase gene controlled by the thymidine kinase promoter. Transient transfection of HEK293, COS-7, U4C, and γ2A cells by Lipofectamine (Invitrogen) was previously described (29). Briefly, cells were seeded in 24-well plates at 2×10^5 cells/well 1 day before transfection. Transfection was carried out according to the manufacturer's recommendations using 250 ng of the appropriate constructs, 500 ng of pGL3-pap1 or pLHRE reporter plasmids, 50 ng of pRLTk control plasmid, and empty vector to normalize total transfected DNA to 1.5 μg. 4 h after transfection cells were stimulated or left unstimulated for 20 h. 24 h after transfection cells were lysed by 150 μl of Passive Lysis Buffer 1× supplied by Promega. Luciferase assays were performed using the dual luciferase reporter assay kit (Promega).

Western Blots—For Western blot analysis, 10^6 HEK293 cells were seeded in 6-well plates 1 day before transient transfection. In brief, HEK293 cells were transfected with 3.75 μg of different constructs depending on the experimental settings using a standard calcium phosphate transfection procedure (Promega). The day after transfection cells were collected and lysed in 250 μl of Laemmli buffer (Bio-Rad) using a 20-gauge syringe. Cellular lysates were boiled for 3 min before loading 20 μl on 12% precast Tris-glycine gels (Invitrogen) and electrophoretically transferred to nitrocellulose membranes (Hybond-C; Amersham Biosciences). After blocking the membranes in 5% milk/Tris-buffered saline-Tween (TBS-Tween), immunoblotting was performed by overnight incubations at 4 °C with primary antibodies in 0.1% TBS-Tween, using the dilution recommended by the manufacturer. After 3 × 5 min of washing in 0.1% TBS-Tween, the membranes were incubated with secondary anti-rabbit-horseradish peroxidase (HRP) or anti-mouse-

HRP antibodies from Cell Signaling Technology (Beverly, MA), diluted 1/5000 in 5% phosphate-buffered saline-TBS-Tween. After 3 × 5 min of washing, a SuperSignal West Pico detection kit (Pierce) and Kodak Biomax Light Film were used for detection. The following phosphospecific antibodies were used: anti-pY1001/1002 JAK1 and anti-pY705 Stat3 (Cell Signaling Technology). Blots were re-probed with anti-JAK1, anti-STAT3 (Cell Signaling Technology), and anti-β-actin (Sigma) antibodies as control.

Immunoprecipitation—For immunoprecipitation, 6×10^6 HEK293 cells were seeded in 100-mm plates 1 day before transfection. The day after, cells were co-transfected using a standard calcium phosphate transfection procedure (Promega) with the 7 μg of Myc-tagged IL-9Rα together with 7 μg of HA-tagged IL-9Rα or HA-tagged EpoR. The day after transfection, cells were collected and lysed in 1 ml of ice-cold modified radioimmune precipitation lysis buffer (1% Nonidet P-40, 0.25% deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), 300 mM NaCl, 1 mM EDTA, 2 mM Na_3VO_4, 1 mM NaF, and complete protease inhibitor mixture tablet (Roche Applied Science) used according to the manufacturer's recommendations) (22) using a 20-gauge syringe and kept 30 min on ice. Clarified lysates were immunoprecipitated with 2 μg of anti-c-Myc antibodies (Santa Cruz) and 20 μl of protein-G-agarose (Roche Applied Science). Immunoprecipitates were boiled, subjected to Tris-glycine gel electrophoresis, and electrotransferred onto nitrocellulose membranes followed by immunoblotting with anti-HA1.1 (Covance) and anti-c-Myc antibody (Santa Cruz). Washing and detection were performed as described above.

FACS Analysis of IL-9Rα Cell Surface Expression—The cell surface expression of the different IL-9Rα constructs was verified by FACS on an aliquot of the cells used for the luciferase assay 1 day after transient transfection. FACS analysis was performed on a BD Biosciences FACSCalibur™ flow cytometer after staining cells with biotinylated anti-human IL-9Rα antibody (22) followed with phycoerythrin-conjugated streptavidin (BD Biosciences) As a control, an aliquot of the transfected cells was stained only with phycoerythrin-conjugated streptavidin (BD Biosciences) antibody.

Reverse Transcription and Quantitative PCR—Total RNA was extracted from the aliquot of the HEK293 cells used for the luciferase assay 1 day after transient transfection. Roughly 5×10^5 cells was used to extract RNA using the TriPure isolation reagent (Roche Applied Science) according to the manufacturer's instructions. Reverse transcription was performed on 1 μg of total RNA with an oligo-(dT) primer (Roche Applied Science) and Moloney murine leukemia virus reverse transcription (Invitrogen). Quantitative PCR reactions were performed using primer sets corresponding to murine JAK1 or human JAK1 with qPCR™ Mastermix for SYBR® Green I (Eurogentec). A series of dilutions of the respective PCR product subcloned into pCR2.1-TOPO vector (Invitrogen) was used a standard for quantification. The sequences of the species-specific JAK1 primers (final concentration, 300 nM) were: mJAK1, 5′-GGAGTGCAGTATCTCTCCTCTCT-3′ (forward) and 5′-CCATGCCCAGGCACTCATTTTCA-3′ (reverse); hJAK1, 5′-TCTTGGAATCCAGTGGAGGCATAAA-3′ (forward) and 5′-CACTCTTCCCGGATCTTGTTTTTCT-3′ (reverse).

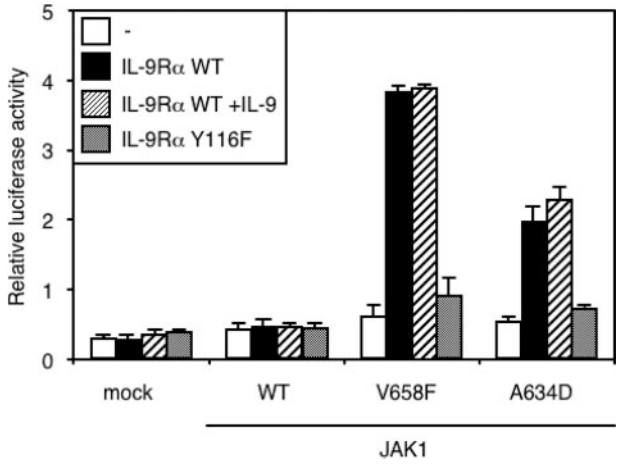


FIGURE 1. **ALL-associated JAK1 mutants (V658F and A634D) induce constitutive STAT3 activation in the presence of IL-9Rα.** HEK293 cells were transiently transfected with different JAK1 constructs alone or in combination with IL-9Rα or the mutated IL-9Rα Y116F. The STAT3-responsive pGL3-pap1 construct was used as luciferase reporter. 4 h post-transfection, cells were incubated for 20 h with or without IL-9 before the luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments.

Samples were first heated for 2 min at 50 °C then 10 min at 95 °C. cDNA was amplified by 40 cycles of a two-step PCR program at 95 °C for 15 s and 60 °C for 1 min. Melting point analysis was carried out by heating the amplicon from 60 to 95 °C.

RESULTS

ALL-associated JAK1 Mutants (V658F and A634D) Induce Constitutive STAT Activation in the Presence of IL-9Rα—ALL-associated JAK1 mutants induce constitutive signaling after ectopic expression in Ba/F3 or BW5147 hematopoietic cells (13). These cells presumably express many receptor complexes that could bind the mutant JAKs and promote constitutive signaling. To study the role of such receptors, we co-expressed components of the IL-9 receptor complex together with JAK1 mutants in HEK293 cells, which are deficient for γc, IL-9Rα, and JAK3. Expression of two ALL-associated JAK1 mutants alone (V658F and A634D) did not induce a significant increase in the level of STAT3 transcriptional activity as assessed by a luciferase assay (Fig. 1). However, co-expression of IL-9Rα with JAK1 mutants (but not WT JAK1) in the absence of JAK3 and γc increased STAT3 activation. Similar results were obtained for STAT5 activation using pLHRE-luc reporter and with the pGRR5-luc reporter, which also responds to STAT1 (data not shown). Stimulation with IL-9 had no effect on IL-9Rα-mediated activation of STAT3 by JAK1 mutants, which is in line with the fact that, in the absence of JAK3 and γc, IL-9 receptor signaling complex cannot support IL-9-induced signal transduction. To prove that mutant JAK1-induced STAT3 activation was dependent on the presence of a functional IL-9Rα, we co-expressed the defective IL-9Rα Y116F, in which the only STAT-recruiting tyrosine residue Tyr-116 was mutated to phenylalanine. We previously showed that this mutation abolishes STAT activation by IL-9 (22). As expected, co-expression of the defective IL-9Rα Y116F did not promote mutant JAK1-induced constitutive STAT3 signaling (Fig. 1), indicating that recruit-

ment of STATs by the IL-9Rα phosphotyrosine was necessary for mutant JAK1-induced constitutive signaling.

These results contrast with a previous report showing that expression of JAK1 V658F in JAK1-deficient U4C fibrosarcoma cells allows for constitutive STAT3 activation (11). To address the hypothesis that this apparent discrepancy was due to the lack of expression of endogenous JAK1 in U4C cells, we measured STAT3 activation in JAK1-deficient U4C or JAK2-deficient γ2A fibrosarcoma cells transfected with wild-type or V658F JAK1. In U4C cells expression of JAK1 V658F alone allowed for constitutive STAT3 activation (8-fold increased luciferase production, as compared with wild-type JAK1). However, co-expression of IL-9Rα chain induced a further 3-fold increase in STAT3 activation (supplemental Fig. 1*A*). By contrast, in γ2A cells expression of JAK1 V658F only marginally increased (1.6-fold) STAT3 transcriptional activity unless the IL-9Rα chain was co-expressed (supplemental Fig. 1*B*). To confirm that JAK1 V658F constitutive activity in U4C cells results from the absence of expression of wild-type JAK1, we cotransfected both isoforms into U4C cells. Co-expression of increasing amounts of wild-type JAK1 together with the JAK1 V658F significantly decreased STAT3 activation (supplemental Fig. 1*C*).

Y107A Mutation in the FERM Domain of JAK1 Mutants Abolishes Constitutive STAT3 Activation—To prove that the JAK1 mutants need to associate to IL-9Rα to constitutively activate STAT3, we mutated tyrosine residue Tyr-107 to alanine in the FERM domain of JAK1 V658F and A634D. Several residues conserved within the FERM domain of JAKs are crucial for cytokine receptor binding (30), and the Y107A mutation within the FERM domain of JAK1 has been shown to abolish JAK1 binding to the gp130 receptor chain (24). As shown in Fig. 2 for two different cell lines (HEK293 and COS-7 cells), the Y107A mutation also abolished the capacity of JAK1 mutants to activate STAT3 in the presence of IL-9Rα. These results indicate that an intact FERM domain of JAK1, essential for the binding to IL-9Rα, is needed for IL-9Rα-mediated constitutive activation of STAT3 by JAK1 mutants.

Co-expression of γc Inhibits IL-9Rα-mediated STAT3 Activation by the JAK1 V658F and A634D Mutants—The functional IL-9 receptor complex is formed by heterodimerization of IL-9Rα and γc, associated with JAK1 and JAK3, respectively. However, our results show that expression of IL-9Rα suffices for mutant JAK1-induced constitutive STAT3 activation. We, therefore, wondered how γc expression would affect this IL-9Rα-mediated constitutive STAT3 activation. Surprisingly, γc expression in the absence of JAK3 blocked IL-9Rα-mediated constitutive activation of STAT3 by JAK1 mutants, as shown by a luciferase assay (Fig. 3*A*). IL-9Rα-mediated activation of STAT5 by JAK1 mutants was modulated by γc in a similar manner (data not shown).

To confirm our observations, we directly studied JAK1 phosphorylation by Western blot using an antibody that detects the phosphorylation of the activation loop Tyr-1022/1023, which reflects the catalytic activity of JAK1. Fig. 3*B* shows that neither wild-type nor JAK1 mutants were phosphorylated when expressed in HEK293 cells alone. As expected, in contrast to wild-type JAK1, co-expression of IL-9Rα with JAK1 V658F or

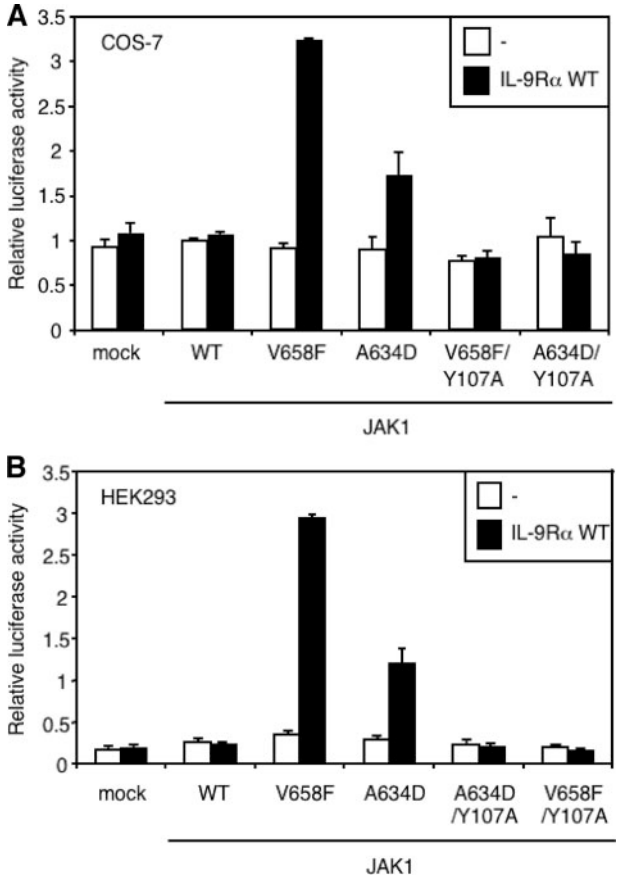


FIGURE 2. **Y107A mutation in the FERM domain of JAK1 mutants abolishes IL-9Rα-mediated constitutive STAT3 activation.** COS-7 (*A*) or HEK293 (*B*) cells were transiently co-transfected with empty vector or different JAK1 constructs with intact or mutated Y107A FERM domain (V658F/Y107A or A634D/Y107A) and with or without IL-9Rα. The STAT3-responsive pGL3-pap1 construct was used as luciferase reporter. 24 h post-transfection cells were subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments in COS-7 and HEK293 cells.

JAK1 A634D increased JAK1 phosphorylation. This IL-9Rα-mediated JAK1 phosphorylation was blocked by co-expression of γc. Phosphorylation of JAK1 was also abolished by the Y107A mutation in the FERM domain. Phosphorylation of STAT3 paralleled JAK1 phosphorylation, confirming the results obtained using the luciferase assay.

A decrease in the expression of IL-9Rα by γc could be an obvious explanation of the γc inhibitory effect. To rule out this hypothesis, we measured the level of IL-9Rα expression by FACS. As shown in Fig. 3*C*, γc expression in HEK293 cells had no effect on IL-9Rα cell surface expression.

Co-expression of JAK3 Partially Reverses the γc Inhibitory Effect on IL-9Rα-mediated Activation of STAT3 by JAK1 V658F—To fully reconstitute the IL-9 receptor complex in HEK293 cells, we tested the effect of JAK3 expression on IL-9Rα-mediated STAT3 activation by JAK1 V658F. As shown in Fig. 4, co-expression of wild-type JAK1 with IL-9Rα alone or with γc did not induce STAT3 activation even after stimulation with IL-9. As expected, the co-expression of JAK3, IL-9Rα, and γc was required for IL-9-induced STAT3 activation. As previously shown, JAK1 V658F activated STAT3 in the presence of IL-9Rα alone, and this activation was inhibited by γc. In the

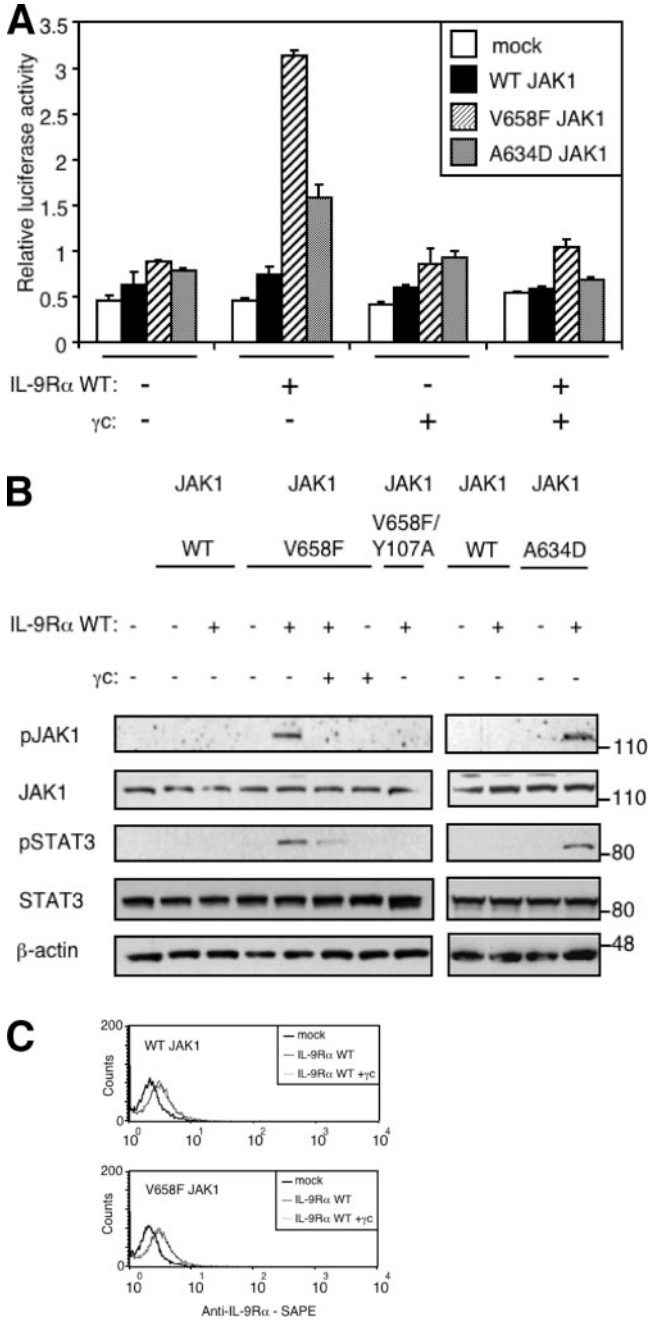


FIGURE 3. **Co-expression of γc inhibits IL-9Rα-mediated JAK1 phosphorylation and STAT3 activation.** *A*, HEK293 cells were transiently co-transfected with empty vector, JAK1 wild-type or V658F, γc, or/and IL-9Rα in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 24 h post-transfection cells were subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments. *B*, HEK293 cells were transiently co-transfected with different empty vector, JAK1 constructs, γc, or/and IL-9Rα. 24 h post-transfection, 10^6 cells were lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT3 was detected using specific anti-pJAK1 Tyr-1022/1023 and anti-pSTAT3 Tyr-705 antibodies. Membranes were reprobed with anti-JAK1, anti-STAT3 and anti-β-actin antibodies as control. Similar results were obtained in two independent experiments. *C*, HEK293 cells were transiently co-transfected with empty vector, JAK1 wild-type or V658F, and IL-9Rα with or without γc in addition to the STAT3-responsive luciferase reporter pGL3-pap1. One day post-transfection, an aliquot of cells was used to assess cell surface expression of IL-9Rα by FACS analysis using anti-human IL-9Rα antibody followed with phycoerythrin-conjugated streptavidin (*SAPE*).

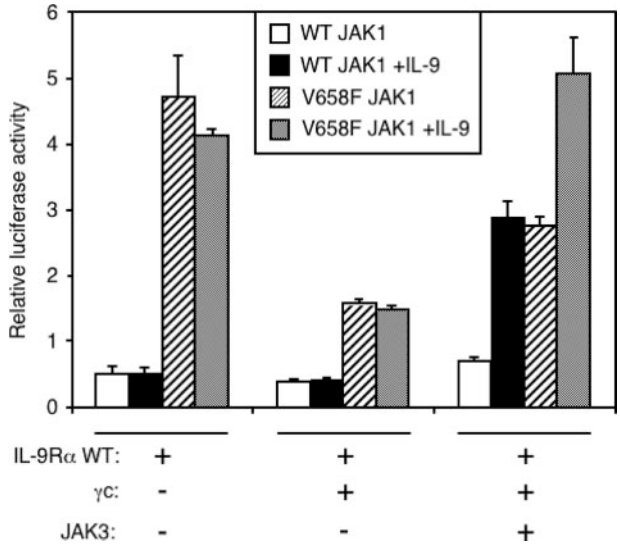


FIGURE 4. **Co-expression of JAK3 partially abrogates the γc inhibitory effect on IL-9Rα-mediated STAT3 activation by JAK1 V658F.** HEK293 cells were transiently co-transfected with JAK1 wild-type or V658F, γc, IL-9Rα, and/or with JAK3 as the last component of IL-9R complex in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 4 h post-transfection cells were incubated for 20 h with or without IL-9 and subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments.

latter situation, JAK3 co-expression partially reversed the inhibitory effect of γc on the IL-9Rα-mediated STAT3 activation by JAK1 V658F. Moreover, in the presence of a functional IL-9 receptor complex, IL-9 was able to increase STAT3 activation above the level obtained for the wild-type JAK1, suggesting that the V658F mutation also potentiates the response to IL-9. These results suggest that, when all the components of the IL-9 receptor complex are expressed, JAK1 V658F and JAK3 cross-activate themselves. Surprisingly, in the absence of IL-9, maximal constitutive activation was obtained with IL-9Rα alone.

Dominant Negative Effect of IL-9Rα BOX1 Mutant on Constitutive JAK1 V658F Phosphorylation and STAT3 Activation Mediated by IL-9Rα—We hypothesized that the constitutive STAT activation observed when JAK1 mutants are co-expressed with the IL-9Rα could be explained by the formation of IL-9Rα homodimers. Indeed, this receptor conformation would allow the cross-phosphorylation of two JAK1 mutants with maximal constitutive activation. In this situation the γc inhibitory effect on IL-9Rα-mediated constitutive signaling could be explained by shifting IL-9Rα homodimeric conformation toward classical heterodimers. To test this hypothesis, we co-expressed WT IL-9Rα with IL-9Rα BOX1 or IL-9Rα IC34 mutants together with JAK1 V658F and looked at the level of STAT3 activation in HEK293 cells by luciferase assay. IL-9Rα BOX1 was obtained by the mutation of two proline residues to serine in the BOX1 motif of IL-9Rα, generating a BOX1-defective IL-9Rα mutant that fails to bind JAK1. IL-9Rα IC34 was obtained after the truncation of the intracellular part of IL-9Rα close to BOX1 residues and was also unable to bind JAK1 (22). Fig. 5*A* shows that these receptors are defective, as their co-expression with JAK1 V658F did not induce constitutive STAT3 activation. Moreover, when co-expressed with IL-9Rα

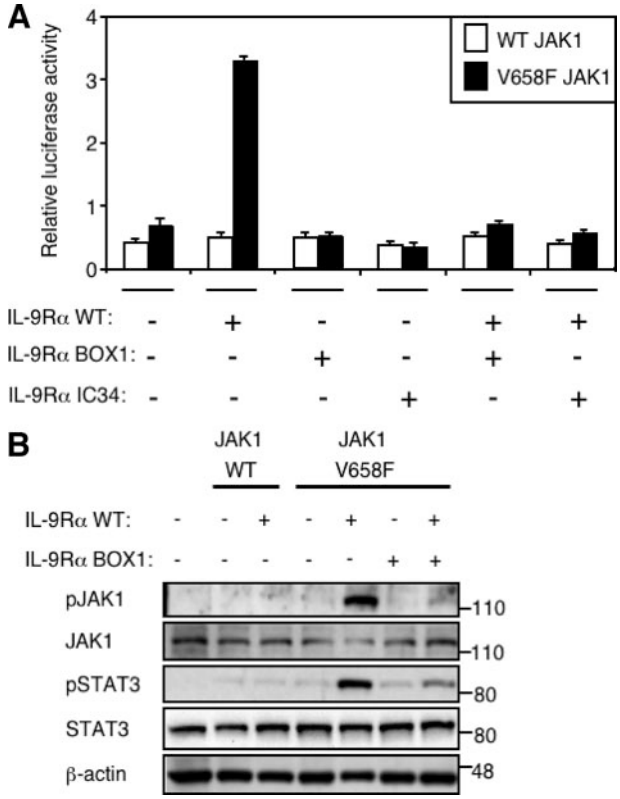


FIGURE 5. **Dominant negative effect of IL-9Rα BOX1 mutant on constitutive JAK1 V658F phosphorylation and STAT3 activation mediated by IL-9Rα WT.** *A*, HEK293 cells were transiently co-transfected with JAK1 wild-type or V658F and different IL-9Rα constructs in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 24 h post-transfection cells were subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments. *B*, HEK293 cells were transiently co-transfected with JAK1 wild-type or V658F and different IL-9Rα constructs. 24 h post-transfection 10^6 cells were lysed and subjected to Western blot analysis. Phosphorylation of JAK1 and STAT3 was detected using specific anti-pJAK1 Tyr-1022/1023 and anti-pSTAT3 Tyr-705 antibodies. Membranes were reprobed with anti-JAK1, anti-STAT3, and anti-β-actin antibodies as control. Similar results were obtained in three independent experiments.

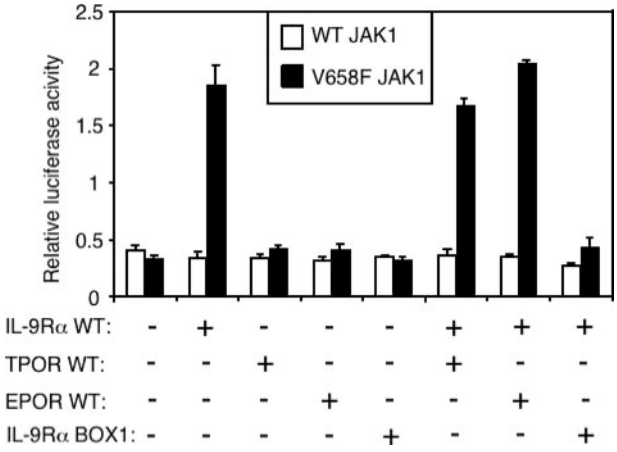


FIGURE 6. **IL-9Rα BOX1 mutant, but not TPOR or EPOR, inhibits IL-9Rα-mediated activation of STAT3 by JAK1 V658F.** HEK293 cells were transiently co-transfected with JAK1 wild-type or V658F, IL-9Rα wild-type, and/or BOX1 mutant or/and with the thrombopoietin or erythropoietin receptors in addition to the STAT3-responsive luciferase reporter. 24 h post-transfection cells were subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in two independent experiments.

WT, these BOX1-defective receptors abolished IL-9Rα-mediated STAT3 activation by JAK1 V658F, exerting a dominant negative effect. This effect is best explained by the disruption of WT IL-9Rα homodimers and the formation of heterodimers between WT and BOX1-defective IL-9Rα. Such heterodimers would not allow cross-activation of JAK1 mutants. We confirmed these results by Western blot analysis of the JAK1 V658F phosphorylation. Fig. 5*B* shows that phosphorylation of JAK1 was present only when JAK1 V658F mutant was co-expressed with IL-9Rα WT but not with IL-9Rα BOX1. Furthermore, co-expression of IL-9Rα BOX1 showed a dominant negative effect on wild-type IL-9Rα-mediated phosphorylation of JAK1 V658F. As expected, STAT3 phosphorylation detected by Western blot paralleled JAK1 phosphorylation (Fig. 5*B*). The expression of wild-type IL-9Rα and different IL-9Rα mutants was measured by FACS and showed similar levels of cell surface expression 24 h after transient transfection of HEK293 cells (supplemental Fig. 2).

In the following experiments we focused on the specificity of the dominant negative effect of IL-9Rα BOX1 on wild-type IL-9Rα-mediated STAT3 constitutive activation by JAK1 V658F. Measuring luciferase activity regulated by a STAT3-specific promoter, we compared the effect of TPOR, EPOR, and IL-9Rα BOX1 mutant expression on this constitutive STAT3 activation in HEK293 cells. As shown in Fig. 6, the dominant negative effect was restricted to IL-9Rα BOX1 mutant, as neither TPOR nor EPOR showed any inhibitory effect on wild-type IL-9Rα-mediated STAT3 activation by JAK1 V658F. Also, in contrast to wild-type IL-9Rα, TPOR and EPOR, which are coupled to JAK2 and not to JAK1, were not able to mediate activation of STAT3 by the JAK1 V658F.

Altogether, these data confirmed the specific dominant negative effect of IL-9Rα BOX1 on IL-9Rα WT-mediated activation of STAT3 by JAK1 V658F supporting our hypothesis of IL-9Rα homodimers. When these homodimers are associated with JAK1 V658F, they promote JAK1 mutant cross-phosphorylation and activation of downstream signaling pathways.

Coimmunoprecipitation Shows Evidence of IL-9Rα Homodimerization—To further confirm IL-9Rα homodimerization, Myc-tagged IL-9Rα and HA-tagged IL-9Rα or EpoR were expressed in HEK293 cells. Cellular extracts were immunoprecipitated with an anti-Myc antibody and analyzed by Western blot with anti-HA antibodies to detect co-immunoprecipitation. As shown in Fig. 7, HA-tagged IL-9Rα, but not HA-tagged EpoR, co-immunoprecipitated with Myc-tagged IL-9Rα when both were expressed in HEK293 cells. HA-tagged IL-9Rα did not co-immunoprecipitate in the absence of Myc-tagged IL-9Rα, confirming specificity of the immunoprecipitation assay and used antibodies. Co-expression of wild-type or V658F JAK1 did not change the pattern of homodimerization (data not shown).

Both IL-2Rβ and IL-9Rα Allow for JAK1 V658F- and JAK1 A634D-induced Constitutive Activation of STAT5—To verify whether the results obtained with IL-9Rα could be extended to other receptors of the same family, we studied the effect of IL-2Rβ expression on JAK1 mutant-induced STAT5 activation.

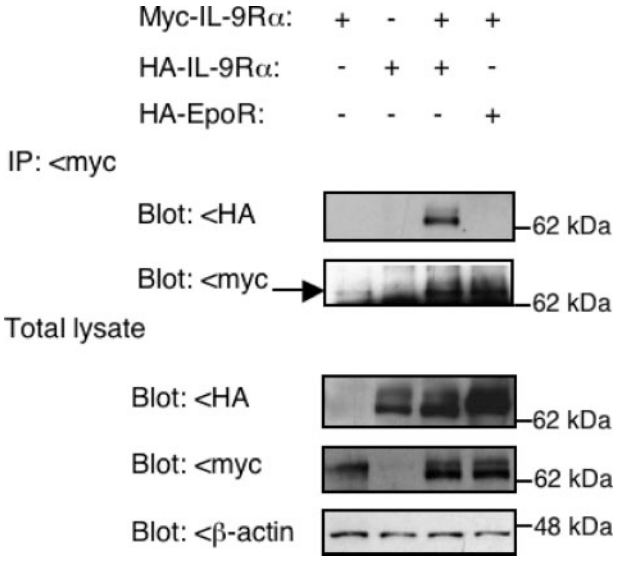


FIGURE 7. **Co-immunoprecipitation shows evidence of IL-9Rα homodimerization.** HEK293 cells were transiently co-transfected with Myc- and/or HA-tagged IL-9Rα or HA-tagged EPOR. 24 h post-transfection, cellular extracts were immunoprecipitated (*IP*) with an anti-Myc antibody and analyzed by Western blot with anti-HA antibodies to detect co-immunoprecipitation of IL-9Rα. Anti-Myc antibodies were used as the control, and total lysates were analyzed with the same antibodies. Similar results were obtained in three independent experiments.

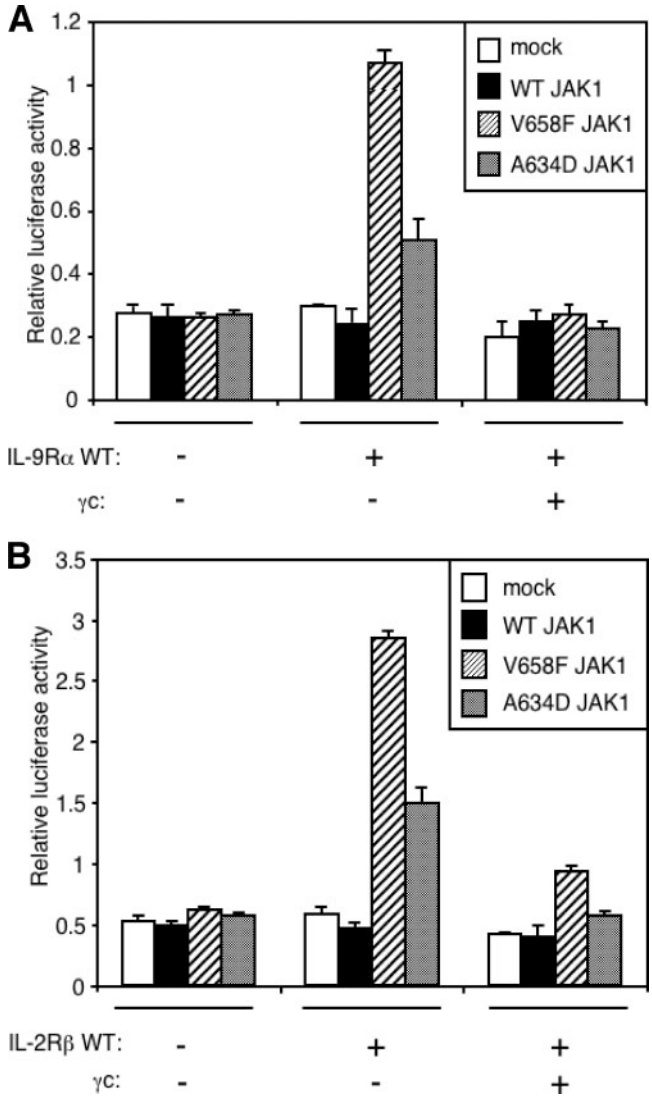


FIGURE 8. **Both IL-9Rα and IL-2Rß allows for JAK1 V658F- and JAK1 A634D-induced constitutive activation of STAT5.** *A*, HEK293 cells were transiently co-transfected with empty vector, JAK1 wild-type, V658F or A634D, IL-9Rα and/or γc in addition to the STAT5-responsive luciferase reporter pLHRE. 24 h post-transfection cells were subjected to a luciferase assay. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments. *B*, the same experiment was performed with IL-2Rβ. Results are the mean ± variation of duplicate samples. Similar results were obtained in three independent experiments.

IL-2 and IL-9 receptor complexes share many similarities. They both require γc, JAK3, and JAK1 for their cytokine-induced signal transduction. However, in contrast to IL-9, IL-2 activates only STAT5. We co-expressed IL-9Rα or IL-2Rβ with JAK1 V658F or A634D in HEK293 cells and assessed STAT5 activity by a luciferase assay. As shown in Fig. 8, IL-2Rβ had the same effect as IL-9Rα and was able to mediate mutant JAK1-induced constitutive STAT5 activation. The formation of IL-2Rß homodimers was strongly suggested by the inhibitory effect of γc co-expression. Altogether, these results show that the mechanism of IL-9Rα homodimerization and mutant JAK1-mediated constitutive activation of STATs can be applied to other cytokine receptor chains from the same cytokine receptor family.

DISCUSSION

In this paper we made two important observations. First, we demonstrate that expression of ALL-associated JAK1 mutants (V658F and A634D) alone does not suffice for constitutive activation of the JAK-STAT pathway but that this process is promoted by JAK1 binding receptors like IL-9Rα or IL-2Rβ. Secondly, we show that IL-9Rα can form homodimers, which mediate JAK1 mutant constitutive activation even better than the functional IL-9 receptor complex.

Recently, the discovery of several mutations in JAK1 in ALL expanded the list of oncogenic JAK mutants, a list led by the JAK2 V617F mutation associated to myeloproliferative neoplasm. In contrast to other oncogenic tyrosine kinases, like BCR-ABL, TEL-JAK2, or FLT3-ITD, JAKs are cytoplasmic tyrosine kinases that cannot form dimers by themselves. For JAK2 V617F, it was demonstrated that association with a homodimeric type I cytokine receptor such as EPOR or TPOR was necessary for constitutive signaling. Preformed dimerization of receptors such as EPOR was proposed to mediate activation or signaling by JAK2 V617F (8). The increased signal mediated by these receptors provides a molecular explanation for the erythrocytosis and thrombocytosis observed in these patients. Therefore, we speculated that JAK1 mutants would also need to associate to receptors to mediate constitutive signaling. Among the different JAK1 binding receptors, we studied the common γ chain family of receptors (IL-2R, -4R, -7R, -9R, -15R, -21R) because they are expressed in B and T cell precursors and because their activation would favor B or T cell proliferation, the phenotype observed in the disease. Indeed, we showed that two of these receptors, IL-9Rα and IL-2Rβ, were able to scaffold mutant JAK1 to mediate constitutive signaling. Moreover, for the IL-9Rα, we demonstrated that the STATs need to be recruited by a phosphotyrosine of the receptor,

showing that the complex of the receptor and its associated JAK mutants has to be considered as the functional oncogenic entity.

In T cell ALL (T-ALL), the IL-2Rβ·JAK1 mutant complex could certainly be oncogenic and explain part of the phenotype. Indeed, IL-2Rβ is expressed in T-ALL (31), and its activation by IL-2 promotes T-cell proliferation and protects T-cells from corticoid-induced apoptosis (32). The expression of IL-9Rα in human T-ALL is controversial, but IL-9 clearly promotes murine T-ALL growth (15, 33). In addition, other complexes of the same family associating mutants JAK1 with IL-4Rα or IL-7Rα, both expressed in most T-ALL (33), are also oncogenic entity candidates.

A particularly intriguing observation was that the IL-9Rα-mediated mutant JAK1 constitutive activation was observed in cells lacking γc and JAK3, necessary for the formation of a functional IL-9 receptor complex. This could be explained by the existence of IL-9Rα monomers able to scaffold JAK1 mutants for activation and able to recruit the STAT factors. However, the dominant negative effect of BOX1 mutants and the co-immunoprecipitation experiments showing the existence of IL-9Rα homodimers argue against that hypothesis. Precedent for preformed dimeric complexes exists for EPOR (10) and for growth hormone receptor (34). Recent experiments from the Henis laboratory (35)using the immunofluorescence co-patching technology also showed that IL-9Rα and IL-2Rβ expressed either transiently in HEK293 cells or stably in BaF3 cells (IL-3-dependent pro B cell line) can form ligand-independent homomeric complexes, but this technology does not allow for identification of the stoichiometry of the complexes. Our data provide direct evidence for dimerization of IL-9Rα, as the dominant negative effect we detect with the IL-9Rα BOX1 mutant would only be consistent for dimers and not higher order complexes (*i.e.* tetramers). Presumably, the dimeric conformation favors the cross-phosphorylation of the two JAK1 mutants associated with IL-9Rα followed by IL-9Rα phosphorylation and STATs activation. One of the JAK1 mutants, V658F, represents the homologous mutation of JAK2 V617F (11), and indeed, such a mechanism was demonstrated for JAK2 V617F, where the homodimeric EPOR conformation was necessary for the constitutive activation (9).

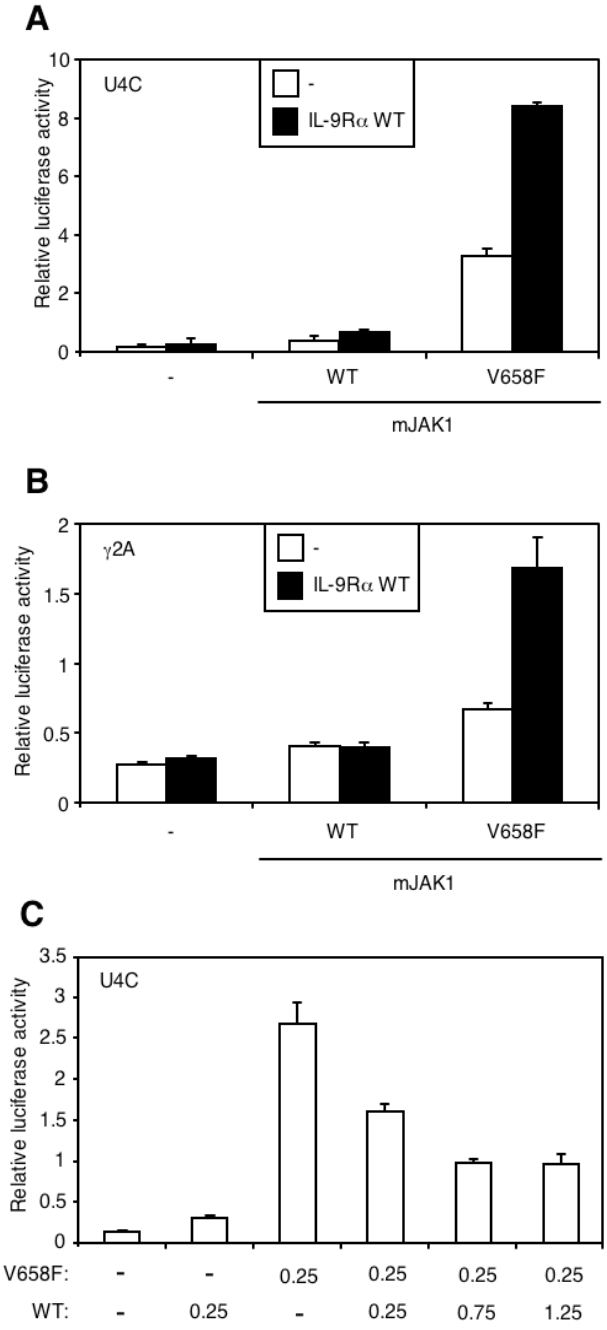
In this model the receptor has a dual role to play; that is, as a scaffold for cross-activation of JAK mutants and as a docking site for the recruitment of the STAT transcription factors. The absence of any potential STAT recruitment site in the cytoplasmic domain of γc implies that this receptor chain is a weak contributor to the oncogenic activity of JAK mutants. Indeed, although γc has the ability to form homodimers, this chain failed to promote STAT activation when associated with the JAK3 A572V mutant unless IL-9Rα or IL-2Rβ were co-expressed (35). According to this model, which STAT is constitutively activated does not depend on the JAK mutant itself but depends on the cytokine receptor associated with a particular JAK mutant. Here, we show constitutive activation of STAT5 by JAK1 mutants associated with IL-9Rα or IL-2Rβ chains, whereas STAT3 activation was detected only in the presence of IL-9Rα. Activation of STAT5 is strongly associated with proliferation and tumoral transformation of hematopoietic progenitors. In contrast, STAT3 activation outcome varies depending on the cellular context and was shown to promote either cell proliferation (36) or differentiation (37). It would be, therefore, important to pay attention to the respective activation of different STATs in ALL expressing JAK1 mutants. Activation of different STAT factors might indeed reflect the involvement of distinct cytokine receptor complexes leading to different oncogenic evolutions.

The fact that signaling by complexes of IL-9Rα and JAK1 mutant without γc and JAK3 was higher than that mediated by the physiological IL-9 receptor complex can be explained by two non-exclusive hypotheses. The first one is that homodimers allow for cross-activation of two JAK1 mutants and increase signaling compared with the physiological JAK1/JAK3 cross-activation. The second one is that homodimers possess a STAT-recruiting phosphotyrosine on each of the IL-9Rα chains, whereas there is only one phosphotyrosine in the functional IL-9 receptor complex. Therefore, in ALL, the level of γc and JAK3 expression, which varies according to the level of B or T cell differentiation and activation, will certainly impact the level of constitutive signaling. Situations might exist when chains like the IL-9Rα or IL-2Rβ might be expressed in excess when compared with γc or to JAK3. Antigen stimulation of naïve T cells results in a strong increase in JAK3 mRNA and protein levels (38, 39) which is followed by an increase in IL-2Rβ and γc levels (40). Future experiments will have to address the possibility that some JAK1 mutant ALL subclones would acquire a proliferative advantage after alteration of the γc gene, which is located on Xq13.1, and therefore expressed from a single allele (41).

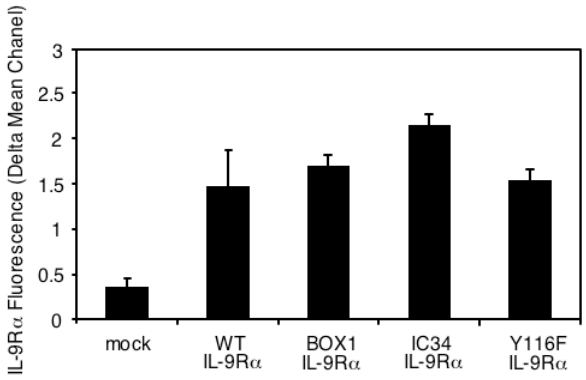
REFERENCES

1. Schindler, C., Levy, D. E., and Decker, T. (2007) *J. Biol. Chem.* **282,** 20059–20063
2. James, C., Ugo, V., Le Couedic, J. P., Staerk, J., Delhommeau, F., Lacout, C., Garcon, L., Raslova, H., Berger, R., Bennaceur-Griscelli, A., Villeval, J. L., Constantinescu, S. N., Casadevall, N., and Vainchenker, W. (2005) *Nature* **434,** 1144–1148
3. Kralovics, R., Passamonti, F., Buser, A. S., Teo, S. S., Tiedt, R., Passweg, J. R., Tichelli, A., Cazzola, M., and Skoda, R. C. (2005) *N. Engl. J. Med.* **352,** 1779–1790
4. Levine, R. L., Wadleigh, M., Cools, J., Ebert, B. L., Wernig, G., Huntly, B. J., Boggon, T. J., Wlodarska, I., Clark, J. J., Moore, S., Adelsperger, J., Koo, S., Lee, J. C., Gabriel, S., Mercher, T., D'Andrea, A., Frohling, S., Dohner, K., Marynen, P., Vandenberghe, P., Mesa, R. A., Tefferi, A., Griffin, J. D., Eck, M. J., Sellers, W. R., Meyerson, M., Golub, T. R., Lee, S. J., and Gilliland, D. G. (2005) *Cancer Cell* **7,** 387–397
5. Baxter, E. J., Scott, L. M., Campbell, P. J., East, C., Fourouclas, N., Swanton, S., Vassiliou, G. S., Bench, A. J., Boyd, E. M., Curtin, N., Scott, M. A., Erber, W. N., and Green, A. R. (2005) *Lancet* **365,** 1054–1061
6. Vainchenker, W., and Constantinescu, S. N. (2005) *Hematology Am. Soc. Hematol. Educ. Program*, **2005,** 195–200
7. Wernig, G., Gonneville, J. R., Crowley, B. J., Rodrigues, M. S., Reddy, M. M., Hudon, H. E., Walz, C., Reiter, A., Podar, K., Royer, Y., Constantinescu, S. N., Tomasson, M. H., Griffin, J. D., Gilliland, D. G., and Sattler, M. (2008) *Blood* **111,** 3751–3759
8. Lu, X., Levine, R., Tong, W., Wernig, G., Pikman, Y., Zarnegar, S., Gilliland, D. G., and Lodish, H. (2005) *Proc. Natl. Acad. Sci. U. S. A.* **102,** 18962–18967
9. Lu, X., Huang, L. J., and Lodish, H. F. (2008) *J. Biol. Chem.* **283,** 5258–5266
10. Constantinescu, S. N., Keren, T., Socolovsky, M., Nam, H., Henis, Y. I., and Lodish, H. F. (2001) *Proc. Natl. Acad. Sci. U. S. A.* **98,** 4379–4384
11. Staerk, J., Kallin, A., Demoulin, J. B., Vainchenker, W., and Constanti-

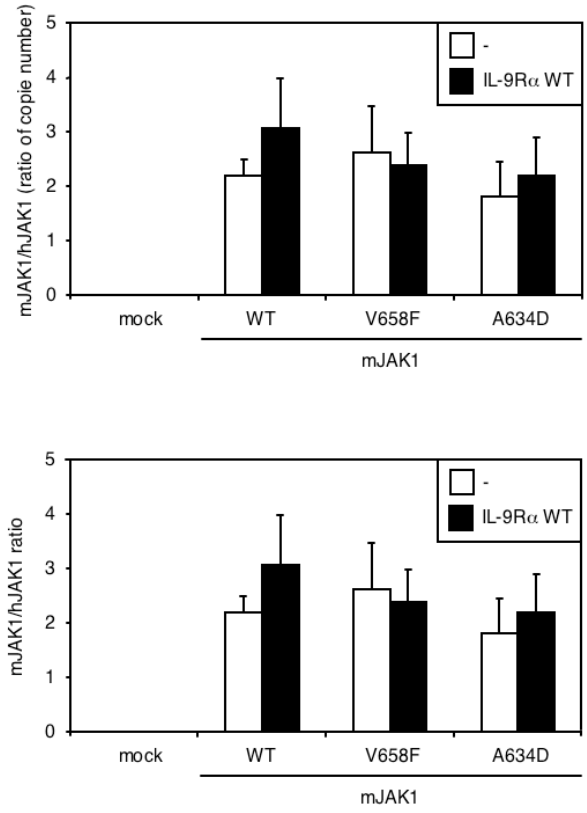
nescu, S. N. (2005) *J. Biol. Chem.* **280,** 41893–41899
12. Walters, D. K., Mercher, T., Gu, T. L., O'Hare, T., Tyner, J. W., Loriaux, M., Goss, V. L., Lee, K. A., Eide, C. A., Wong, M. J., Stoffregen, E. P., McGreevey, L., Nardone, J., Moore, S. A., Crispino, J., Boggon, T. J., Heinrich, M. C., Deininger, M. W., Polakiewicz, R. D., Gilliland, D. G., and Druker, B. J. (2006) *Cancer Cell* **10,** 65–75
13. Flex, E., Petrangeli, V., Stella, L., Chiaretti, S., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., Martinelli, S., Fragale, A., Sanchez, M., Tavolaro, S., Messina, M., Cazzaniga, G., Camera, A., Pizzolo, G., Tornesello, A., Vignetti, M., Battistini, A., Cave, H., Gelb, B. D., Renauld, J. C., Biondi, A., Constantinescu, S. N., Foa, R., and Tartaglia, M. (2008) *J. Exp. Med.* **205,** 751–758
14. Jeong, E. G., Kim, M. S., Nam, H. K., Min, C. K., Lee, S., Chung, Y. J., Yoo, N. J., and Lee, S. H. (2008) *Clin. Cancer Res.* **14,** 3716–3721
15. Renauld, J. C., van der Lugt, N., Vink, A., van Roon, M., Godfraind, C., Warnier, G., Merz, H., Feller, A., Berns, A., and Van Snick, J. (1994) *Oncogene* **9,** 1327–1332
16. Merz, H., Houssiau, F. A., Orscheschek, K., Renauld, J. C., Fliedner, A., Herin, M., Noel, H., Kadin, M., Mueller-Hermelink, H. K., Van Snick, J., and Feller, A. C. (1991) *Blood* **78,** 1311–1317
17. Fischer, M., Bijman, M., Molin, D., Cormont, F., Uyttenhove, C., van Snick, J., Sundstrom, C., Enblad, G., and Nilsson, G. (2003) *Leukemia* **17,** 2513–2516
18. Kelleher, K., Bean, K., Clark, S. C., Leung, W. Y., Yang-Feng, T. L., Chen, J. W., Lin, P. F., Luo, W., and Yang, Y. C. (1991) *Blood* **77,** 1436–1441
19. Uyttenhove, C., Druez, C., Renauld, J. C., Herin, M., Noel, H., and Van Snick, J. (1991) *J. Exp. Med.* **173,** 519–522
20. Demoulin, J. B., Uyttenhove, C., Lejeune, D., Mui, A., Groner, B., and Renauld, J. C. (2000) *Cancer Res.* **60,** 3971–3977
21. Knoops, L., and Renauld, J. C. (2004) *Growth Factors* **22,** 207–215
22. Demoulin, J. B., Uyttenhove, C., Van Roost, E., DeLestre, B., Donckers, D., Van Snick, J., and Renauld, J. C. (1996) *Mol. Cell. Biol.* **16,** 4710–4716
23. Druez, C., Coulie, P., Uyttenhove, C., and Van Snick, J. (1990) *J. Immunol.* **145,** 2494–2499
24. Haan, C., Is'harc, H., Hermanns, H. M., Schmitz-Van De Leur, H., Kerr, I. M., Heinrich, P. C., Grotzinger, J., and Behrmann, I. (2001) *J. Biol. Chem.* **276,** 37451–37458
25. Liu, X., Constantinescu, S. N., Sun, Y., Bogan, J. S., Hirsch, D., Weinberg, R. A., and Lodish, H. F. (2000) *Anal. Biochem.* **280,** 20–28
26. Renauld, J. C., Druez, C., Kermouni, A., Houssiau, F., Uyttenhove, C., Van Roost, E., and Van Snick, J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 5690–5694
27. Gerland, K., Bataille-Simoneau, N., Basle, M., Fourcin, M., Gascan, H., and Mercier, L. (2000) *Mol. Cell. Endocrinol.* **168,** 1–9
28. Peelman, F., Iserentant, H., De Smet, A. S., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2006) *J. Biol. Chem.* **281,** 15496–15504
29. Dumoutier, L., Van Roost, E., Colau, D., and Renauld, J. C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 10144–10149
30. Saharinen, P., Takaluoma, K., and Silvennoinen, O. (2000) *Mol. Cell. Biol.* **20,** 3387–3395
31. Baba, H., Yamada, Y., Mori, N., Hayashibara, T., Harasawa, H., Tsuruda, K., Sugahara, K., Soda, H., Takasaki, Y., Tawara, M., Hirakata, Y., Tomonaga, M., and Kamihira, S. (2002) *Eur. J. Haematol.* **68,** 362–369
32. Renauld, J. C., Vink, A., Louahed, J., and Van Snick, J. (1995) *Blood* **85,** 1300–1305
33. Vink, A., Renauld, J. C., Warnier, G., and Van Snick, J. (1993) *Eur. J. Immunol.* **23,** 1134–1138
34. Brown, R. J., Adams, J. J., Pelekanos, R. A., Wan, Y., McKinstry, W. J., Palethorpe, K., Seeber, R. M., Monks, T. A., Eidne, K. A., Parker, M. W., and Waters, M. J. (2005) *Nat. Struct. Mol. Biol.* **12,** 814–821
35. Malka, Y., Hornakova, T., Royer, Y., Knoops, L., Renauld, J. C., Constantinescu, S. N., and Henis, Y. I. (2008) *J. Biol. Chem.* **283,** 33569–33577
36. Hideshima, T., Bergsagel, P. L., Kuehl, W. M., and Anderson, K. C. (2004) *Blood* **104,** 607–618
37. Bromberg, J., and Darnell, J. E., Jr. (2000) *Oncogene* **19,** 2468–2473
38. Kawamura, M., McVicar, D. W., Johnston, J. A., Blake, T. B., Chen, Y. Q., Lal, B. K., Lloyd, A. R., Kelvin, D. J., Staples, J. E., Ortaldo, J. R., and O'Shea, J. J. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 6374–6378
39. Johnston, J. A., Kawamura, M., Kirken, R. A., Chen, Y. Q., Blake, T. B., Shibuya, K., Ortaldo, J. R., McVicar, D. W., and O'Shea, J. J. (1994) *Nature* **370,** 151–153
40. Nakarai, T., Robertson, M. J., Streuli, M., Wu, Z., Ciardelli, T. L., Smith, K. A., and Ritz, J. (1994) *J. Exp. Med.* **180,** 241–251
41. Noguchi, M., Yi, H., Rosenblatt, H. M., Filipovich, A. H., Adelstein, S., Modi, W. S., McBride, O. W., and Leonard, W. J. (1993) *Cell* **73,** 147–157



SUPPLEMENTARY FIGURE 1 Co-expression of wild-type JAK1 inhibits ability of JAK1 V658F to constitutively activate STAT3. *A) JAK1-deficient U4C fibrosarcoma cells were transiently transfected with empty vector, JAK1 wild-type or V658F with or without IL-9Rα (in the absence of JAK3 and γc chain), in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 24 hours post-transfection, cells subjected to a luciferase assay. Results are mean ± S.D. of triplicate samples. Similar results were obtained in 2 independent experiments. B) The same experiment was performed with JAK2-deficient γ2A human fibrosarcoma cell line. Results are mean ± S.D. of triplicate samples. Similar results were obtained in 2 independent experiments. C) JAK1-deficient U4C human fibrosarcoma cells were transiently transfected with 0.25 μg of empty vector, JAK1 wild-type and V658F alone or co-transfected with increasing concentration of wild-type JAK1 (0.25, 0.75 or 1.25 μg), in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 24 hours post-transfection, cells were subjected to a luciferase assay. Results are mean ± S.D. of triplicate samples. Similar results were obtained in 2 independent experiments.*



SUPPLEMENTARY FIGURE 2 The cell surface expression of wild-type and mutated IL-9Rα constructs in HEK293 cells. *HEK293 cells were transiently transfected with empty vector, wild-type or mutated IL-9Rα constructs. One day after transient transfection, an aliquot of the cells was used to assess cell surface expression of IL-9Rα by FACS analysis using biotinylated anti-human IL-9Rα antibody followed with phycoerythrin-conjugated Streptavidin. The rest of the transfected cells was stained only with phycoerythrin-conjugated Streptavidin as a control. After FACS analysis was performed, the geometric mean channel of the fluorescence of anti-IL-9Rα-SAPE stained and SAPE-only stained cells was compared and used to calculate the delta mean channel. Results are mean ± variation of duplicate transfections.*



SUPPLEMENTARY FIGURE 3 The expression of wild-type and mutated murine JAK1 in human HEK293 cells. *HEK293 cells were transiently transfected with empty vector, wild-type, V658F or A634D murine JAK1 with or without IL-9Rα chain, in addition to the STAT3-responsive luciferase reporter pGL3-pap1. 24 hours post-transfection, an aliquot of cells was used for the RNA extraction and cDNA synthesis. Quantitative PCR was performed using mouse or human JAK1-specific primers and a series of dilutions of the respective clones cDNA as a standard. Results are mean ± variation of duplicate transfections. Similar results were obtained in 2 independent experiments.*

ALL-associated JAK1 Mutations Confer Hypersensitivity to the Anti-Proliferative Effect of Type I Interferon

Tekla Hornakova, Sabina Chiaretti, Muriel M. Lemaire, Robin Foà, Raouf Ben Abdelali, Vahid Asnafi, Marco Tartaglia, Jean-Christophe Renauld and Laurent Knoops

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Preamble

In the preceding parts of this thesis, we showed that JAK1 mutations occur in acute lymphoblastic leukemia (ALL), and that JAK1 mutants need to associate to cytokine receptors, such as IL-2R and IL-9R, to promote constitutive activation of the receptor complex and signal transduction via STAT proteins (169). However, beside the common gamma chain-related cytokine receptor subfamily, JAK1 participates in the signal transduction of other cytokine receptor families such as the IFN receptor complexes.

Type I IFNs (IFN-α/β) are important cytokines involved in the defense against viral infection (170). In contrast to most JAK1-activating cytokines, that promote cell proliferation and differentiation, type I IFN rather induce inhibition of cell proliferation and promote apoptosis (171).

The anti-proliferative effect of IFN-α is used in the clinic to treat patient with myeloproliferative neoplasms (MPNs), melanoma or renal cell carcinoma (172,173). However, interferon is not used to treat ALL (140).

In this study we show that different JAK1 mutants potentiate the anti-proliferative effect of type I IFNs *in vitro* and *in vivo*. Thus, our data suggest that IFN-α might represent a candidate as adjuvant targeted therapy for ALL patients harboring JAK1 mutations.

ALL-associated *JAK1* Mutations Confer Hypersensitivity to the Anti-Proliferative Effect of Type I Interferon

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ABSTRACT

Activating mutations in *JAK1* have been reported in acute lymphoblastic leukemias (ALL). In this study, we found a type I IFN transcriptional signature in *JAK1* mutation-positive human ALL samples. This signature was recapitulated *in vitro* by the expression of JAK1 mutants in BW5147 and BaF3 hematopoietic cell lines. Binding of JAK1 to the IFN receptor was essential since mutations in the FERM domain abrogated this effect. Beside the constitutive activation of the type I IFN signaling cascade, *JAK1* mutations also strongly potentiated the response to IFN *in vitro*. Typically, the proliferation of cell lines expressing $JAK1^{A634D}$ was abrogated by type I IFNs. Interestingly, we found that different *JAK1* mutations differentially potentiate responses to type I IFNs or to IL-9, another cytokine using JAK1 to mediate its effects. This suggests that the type of mutation influences the specificity of the effect on distinct cytokine receptor signaling. Finally, we also showed in an *in vivo* leukemia model that cells expressing $JAK1^{A634D}$ are hypersensitive to the anti-proliferative and anti-tumorigenic effect of type I IFN, suggesting that type I IFNs should be considered as a potential therapy for ALL with *JAK1* activating mutations.

INTRODUCTION

JAK1 is a member of the Janus kinase family comprising four non-receptor tyrosine kinases (JAK2, JAK3 and TYK2) that associate to cytokine receptors without intrinsic kinase activity to mediate cytokine-induced signal transduction (1). Recently, we and others reported that JAK1 mutations occur in acute lymphoblastic leukemia (ALL), and are relatively common in adult patients with T cell ALL (2-4). Some of these mutations, such as A634D and R724H, induce the autonomous growth of the cytokine-dependent BaF3 cell line, whereas the R897C and A634D mutants protect the murine ALL cell line BW5147 from dexamethasone-induced apoptosis, indicating that they represent gain of function mutations that participate in the leukemogenic process by inducing the constitutive activation of the JAK-STAT pathway (2).

JAKs participate in the signal transduction of various cytokine receptor families, which regulate different cellular processes. JAK1-binding receptors include the common gamma chain subfamily (IL-2R, IL-4R, IL-7R, IL-9R, IL-15R, IL-21R), the gp130 subfamily (IL-6R, LIFR, IL-11R) and the type II cytokine receptor complexes (type I, II and III IFN receptors, IL-10R). We showed that JAK1 mutants need to associate to cytokine receptors, such as IL-2R and IL-9R, to promote constitutive activation of the receptor complex and signal transduction via signal transducers and activators of transcription, STATs (5). A similar

mechanism was demonstrated for the JAK2 V617F mutant found in myeloproliferative neoplasms. JAK2 V617F needs to associate to homodimeric type I cytokine receptors, such as the erythropoietin or thrombopoietin receptor, to mediate the cytokine receptor-JAK2-STAT5 constitutive activation responsible for the increased myeloproliferation (6,7).

Type I interferons (IFN-α/β) are important cytokines involved in the defense against viral infection. The type I IFN receptor complex consists of two receptor subunits, IFNAR1 and IFNAR2, which are associated with TYK2 and JAK1 respectively (8). Ligand-induced stimulation of IFNAR results in cross-activation of TYK2 and JAK1, which in turn activate two members of the STAT family, STAT1 and STAT2 (9). Activation of STAT1 and STAT2 leads to the formation of two transcriptional complexes. The first complex, IFN-α-activated factor, is a homodimer of activated STAT1. The second complex, IFN-stimulated gene factor 3 (ISGF3), consists of a complex of activated STAT2, STAT1 and IRF-9 (a member of interferon regulatory factors, IRFs). STAT1 activation is involved in signaling from various cytokine receptors (9), whereas activation of STAT2 and formation of the ISGF3 complex are specifically triggered by type I IFN receptor complex and in a small number of cell types by type III IFN receptor complex (10,11). In contrast to other JAK1-binding cytokine receptors, type I IFN receptors are ubiquitously expressed (8) and ligand-induced activation of type I IFN receptor complex was shown to induce inhibition of cell proliferation and apoptosis. Expression of anti-proliferative and pro-apoptotic genes was showed to be mainly under the control of the ISGF3 complex, e.g. STAT2 and IRF-9 (12). The anti-proliferative effect of interferon-α is used in the clinic to treat patient with myeloproliferative neoplasms, melanoma or renal cell carcinoma (13,14).

In this study, we found that tumor cells from patients with *JAK1* mutation-positive ALL exhibit a type I IFN signature. *In vitro*, we showed that several JAK1 mutants constitutively activate components of type I IFN receptor signaling cascade, such as TYK2, STAT2 and STAT1. Moreover, cell lines expressing JAK1 mutants were hypersensitive to the anti-proliferative effect of type I IFNs, which in turn resulted in a better response to the anti-tumorigenic effect of IFN in an *in vivo* leukemia model.

RESULTS

A type I IFN transcriptional signature characterizes JAK1 mutation-positive ALL. To identify the signaling pathways regulated by constitutively active mutations in *JAK1*, we performed a geneset enrichment analysis (GSEA) based on the genes differentially expressed by 3 *JAK1* mutation-positive T-ALL samples compared to 8 *JAK1* mutation-negative T-ALL samples. GSEA is a computational method for determining whether a rank-ordered list of genes for a particular comparison of interest (e.g. samples with mutated versus wild-type *JAK1*) is enriched in genes derived from an independently generated gene set (e.g., genes induced by IFN-a in primary hepatocytes). Among the 1,892 predefined genesets available from the curated Molecular Signature Database (http://www.broad.mit.edu/gsea/msigdb/index.jsp), the GSEA analysis revealed that 20 genesets were significantly overexpressed in the *JAK1* mutation-positive cases compared to the samples without a mutated *JAK1* allele. As shown in Figure 1A for the 10 most enriched genesets, the vast majority of these genesets contained either type I IFN-induced genes or were composed of genes found in situations where type I IFN can be produced to activate IFN-target genes, like in systemic lupus erythematosus (SLE). Figure 1B shows a typical GSEA enrichment plot illustrating the enrichment in IFN-target genes among those that were upregulated in *JAK1* mutation-positive ALL blasts. These results suggested that *JAK1* mutation-positive T-ALL cells were characterized by the activation of pathways leading to the expression of type I IFN target genes. Based on this observation, we hypothesized that clustering of our ALL samples based on another IFN target gene signature should clearly distinguish those with or without a *JAK1* mutation. Focusing on genes induced by IFN in hematopoietic cells, we selected a list of genes demonstrated to be induced by IFN in PBMC (15). As shown in Figure 1C, hierarchical clustering based on this signature perfectly separated *JAK1* mutation-positive ALL cases from those without a *JAK1* mutation. To confirm these observations, we used an independent set of 6 *JAK1* mutation-positive and 11 mutation-negative T-ALL samples (16) to assess the expression of two IFN target genes, IRF-7 and MX1. These genes are good surrogate of the IFN signature and were overexpressed in the first cohort of JAK1 mutated samples based on the microarray data (Figure 1D). As shown in Figure 1E, both IFN-target genes were significantly upregulated in the 6 independent *JAK1* mutation-positive compared to 11 mutation-negative T-ALL samples. Altogether, these data show that *JAK1* mutation-positive T-ALL cells are characterized by a type I IFN transcriptional signature.

A

NAME	FWER p-val	Brief description
TAKEDA_NUP8_HOXA9_3D_UP	<0.001	Effect of NUP98-HOXA9 on gene transcription at 3 d after transduction UP
RADAEVA_IFNA_UP	<0.001	Genes up-regulated by interferon-alpha in primary hepatocyte
IFNALPHA_HCC_UP	<0.001	Upregulated by interferon alpha treatment in Hep3B hepatocellular carcinoma cells
SANA_IFNG_ENDOTHELIAL_UP	<0.001	Genes up-regulated by interferon-gamma in colon,derm,iliac,aortic,lung endothelial cells
BENNETT_SLE_UP	<0.001	Genes Significantly up-regulated in SLE patient Blood Mononuclear Cells
IFNALPHA_NL_UP	<0.001	Upregulated by interferon alpha treatment in normal primary hepatocytes
IFNA_HCMV_6HRS_UP	<0.001	Up-regulated in fibroblasts at 6 hours following treatment with interferon-alpha
DER_IFNA_UP	<0.001	Genes up-regulated by interferon-alpha in HT1080 (fibrosarcoma)
TAKEDA_NUP8_HOXA9_8D_UP	<0.001	Effect of NUP98-HOXA9 on gene transcription at 8 d after transduction UP
DER_IFNB_UP	<0.001	Genes up-regulated by interferon-beta in HT1080 (fibrosarcoma)

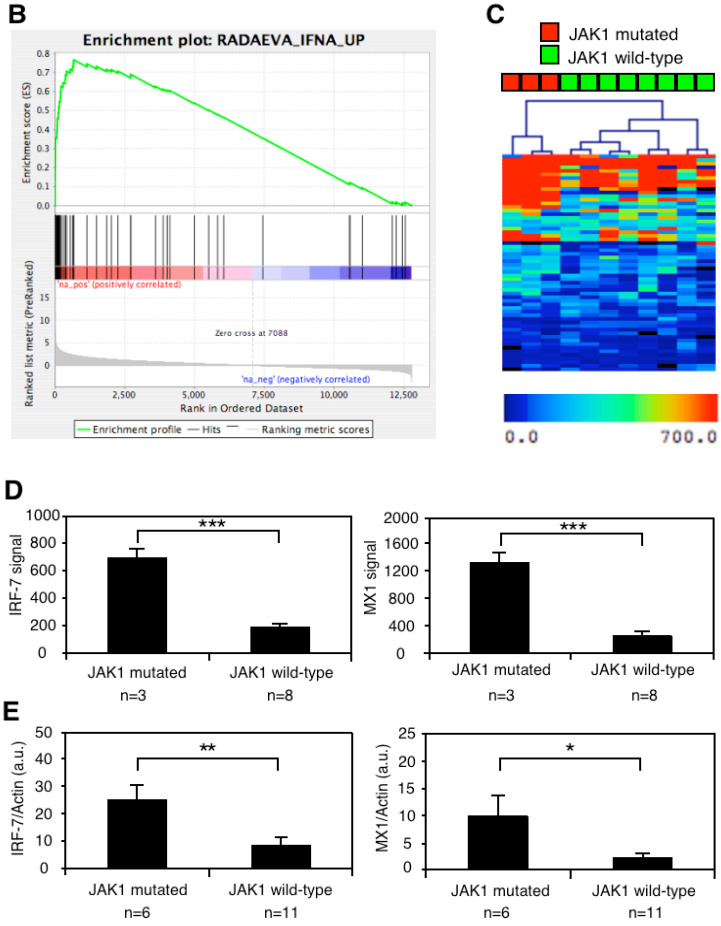

FIGURE 1 JAK1 mutation-positive T-ALL cells show a type I IFN transcriptional signature. *The gene expression profile of human JAK1 mutation-positive and mutation-negative T-ALL cells was assessed with pangenomic microarrays. (A) The list of the 10 genesets most enriched in cells with a mutated JAK1 allele, according to a GSEA analysis is shown. (B) The figure represents the GSEA enrichment plot of one of the most enriched geneset. The lower part of this plot shows the ranking of the genes relative to their enrichment in JAK1 mutation-positive cells. The medium part shows the ranking of the hits from the IFN-induced geneset and the top part shows the resulting enrichment profile. (C) Hierarchical clustering based on the genes induced by IFN-α in PBMC. (D) The mean microarray signal for the IFN targets hIRF-7 (right panel) and hMX1 (left panel) is represented for 3 JAK1 mutation-positive compared to 8 mutation-negative T-ALL samples. The differences between JAK1 mutation-positive and mutation-negative samples were extremely significant (***p<0.0001; t test). Results are mean ± SEM. (E) Quantitative analysis hIRF-7 (left panel) and hMX1 (right panel) expression, based on an independent set of 6 JAK1 mutation-positive and 11 mutation-negative T-ALL samples. Transcripts were normalized to the level of the β-Actin transcripts. The differences between JAK1 mutation-positive and mutation-negative ALL samples were significant (**p=0.0071, *p=0.0348; t test). Results are mean ± SEM.*

The JAK1^{A634D} mutant, but not JAK1$^{A634D/Y107A}$, mediates constitutive expression of type I IFN-regulated genes and promotes constitutive phosphorylation of the components of the type I IFN signal transduction pathway in BW5147 and BaF3 cells. We previously reported that, at physiological level, the ALL-associated JAK1 mutants need to associate to cytokine receptors to allow for constitutive signaling (5). In this model of JAK1 mutant activation, the receptor has a dual role to play: as a scaffold for cross-activation of JAKs and as a docking site for the STAT transcription factors. Activation of different STAT transcription factors and expression of STAT-regulated genes might therefore reflect the involvement of multiple cytokine receptor complexes, each with a distinct perturbing effect on gene expression (5). Based on the interferon signature observed in *JAK1* mutation-positive ALL samples, we hypothesized that ALL-associated JAK1 mutants bind to type I IFN receptor complex and constitutively

activate type I IFN receptor signal transduction. To address this hypothesis, we stably expressed JAK1^{A634D}, one of the ALL-associated JAK1 mutants, in BW5147, a murine T-ALL cell line, and BaF3, a murine pro-B cell line by retroviral infection. The level of expression of IRF-7 and Oasl-2, two specific type I IFN-regulated genes, were assessed by quantitative RT-PCR in cells transduced by JAK1^{WT} and JAK1^{A634D}, with or without the FERM domain mutation Y107A, which is known to abolish JAK1 binding to receptors. As shown in Figure 2A and 2B, expression of the JAK1^{A634D} mutant promoted constitutive expression of IRF-7 and Oasl-2. As expected, the FERM domain mutation abolished this effect, supporting the idea that JAK1 binding to the receptor is required for the activating effect of the *JAK1* mutation on signaling (5). Expression of the JAK1^{A634D} mutant in BaF3 allows for selection of IL-3-independent autonomous cells, which was associated with constitutive STAT5 activation (2). As shown in Figure 2, these autonomously growing cells expressed even higher level of IRF-7 and Oasl-2 than JAK1^{A634D} expressing BaF3 non-autonomous cells proliferating in a medium containing IL-3, suggesting a stronger activation of type I IFN signal transduction in the former. To dissect the mechanism of constitutive expression of type I IFN-regulated genes in JAK1^{A634D} expressing BaF3 cells, we studied the activation status of proteins involved in the signal transduction of type I IFN receptor complex. We observed that JAK1^{A634D} was constitutively phosphorylated in BaF3. Interestingly, phosphorylation of TYK2 paralleled the JAK1 phosphorylation pattern (Figure 2C). Because TYK2 and JAK1 are respectively associated with IFNAR1 and IFNAR2, this suggested that TYK2 represents a partner of JAK1 in mediating the type I IFN-stimulated signal, and is cross-phosphorylated and activated by JAK1^{A634D}. Constitutive phosphorylation of STAT1 and of STAT2, which is a specific feature of the type I IFN receptor complex activation, was also detected in BaF3 cells expressing the JAK1 mutant, confirming constitutive activation of the pathway. Phosphorylation and activation of all these signaling molecules was increased after autonomous selection. Of note, total STAT2 protein level was also increased in cells expressing JAK1^{A634D} (Figure 2C), in line with previous observations showing that STAT2 expression is upregulated upon IFN stimulation (17). Constitutive phosphorylation of JAK1, STAT1 and STAT2 was also observed in JAK1^{A634D} expressing BW5147 cells (data not shown).

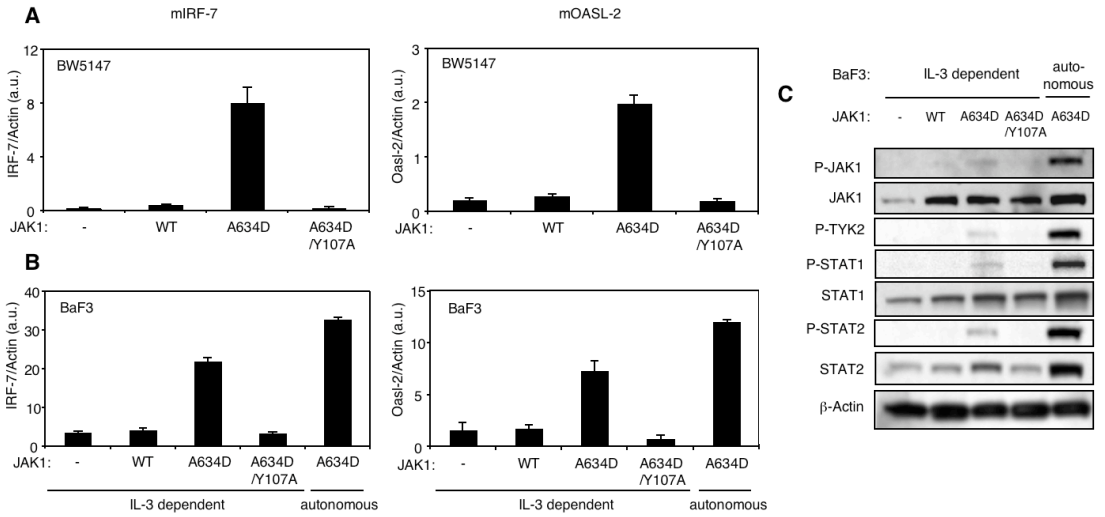

FIGURE 2 The JAK1^{A634D} mutant, but not JAK1$^{A634D/Y107A}$, mediates constitutive expression of type I IFN-regulated genes and promotes constitutive phosphorylation of the components of the type I IFN signal transduction pathway in BW5147 and BaF3 cells. *Total RNA was extracted from 10^6 BW5147 (A) and BaF3 (B) cells stably transduced by JAK1^{WT} or JAK^{A634D} with intact or mutated Y107A FERM domain (JAK1$^{A634D/Y107A}$). To quantify basal expression of type I IFN-induced genes, such as mIRF-7 (left panel) and mOasl-2 (right panel), quantitative RT-PCR was performed. Transcripts were normalized to the level of the murine β-Actin transcripts. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments. (C) 10^6 BaF3 cells stably transduced with JAK1^{WT} or JAK^{A634D}, with intact or mutated Y107A FERM domain (JAK1$^{A634D/Y107A}$), were starved for 4 hours, lyzed and subjected to Western Blot analysis. Basal phosphorylation of JAK1, TYK2, STAT1 and STAT2 was detected using specific anti-pY1022/1023 JAK1, anti-pY1054/1055 TYK2, anti-pY701 STAT1 and anti-pY689 STAT2 antibodies. Membranes were reprobed with anti-JAK1, anti-STAT1, anti-STAT2 and anti-β-Actin antibodies as control. Similar results were obtained in 3 independent experiments for the BaF3 and BW5147 cell lines.*

JAK1^{A634D} upregulates type I IFN responsiveness. The V678F amino acid substitution in TYK2, the IFNAR1-associated kinase, has been previously shown to constitutively activate STAT3 without affecting ligand-induced signaling through the type I IFN receptor (18). We investigated whether the JAK1^{A634D} mutant could enhance type I IFN responsiveness. Figure 3A shows that expression of JAK1^{A634D} not only promoted constitutive transcription of type I IFN-regulated genes (IRF-7 and Oasl-2), but also significantly upregulated IFN-β-dependent induction of these genes. To confirm that the JAK1 mutant upregulates ligand-induced signaling through the type I IFN receptor, we took advantage of the luciferase reporter construct, pIRF7-luc, which uses the ISGF3-responsive IRF-7 promoter to control luciferase transcription. As shown on Figure 3B, reporter transactivation was increased in all IFN-β-stimulated BW5147 cells, but this induction was 3-4 fold higher in cells expressing JAK1^{A634D}. Consistent results were obtained in BaF3 cells (data not shown). Overall, these data strongly suggest that, in contrast to the TYK2^{V678F} mutant, the ALL-associated JAK1^{A634D} mutant potentiates ligand-induced signaling through the type I IFN receptor complex.

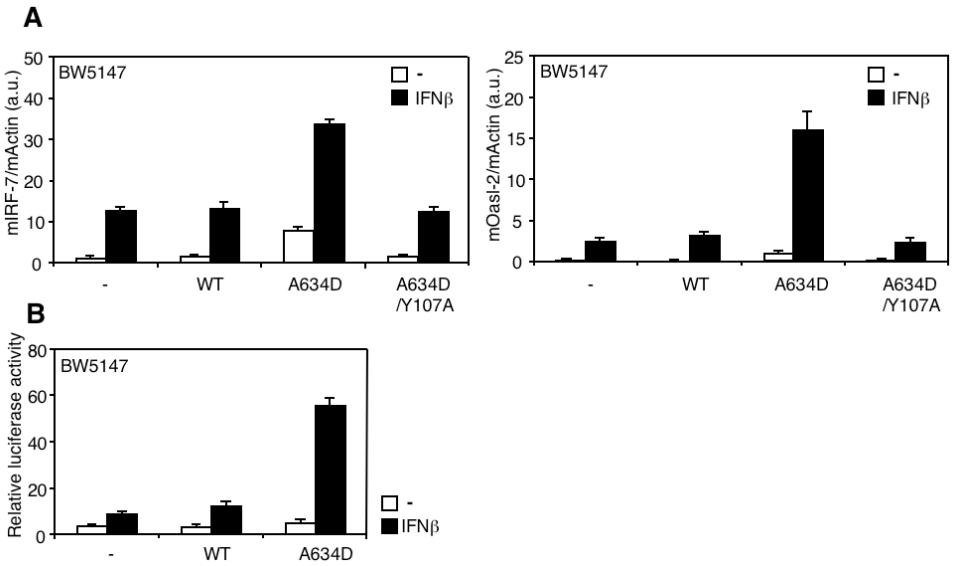

FIGURE 3 JAK1^{A634D} upregulates ligand-induced signaling through the type I IFN receptor. *A) 10^6 BW5147 cells stably transduced with JAK1^{WT} or JAK^{A634D}, with intact or mutated Y107A FERM domain (JAK1$^{A634D/Y107A}$), were stimulated for 3 hours with 1% IFN-β or mock/control HEK293 supernatant, lyzed and used for total RNA extraction. To quantify ligand-induced expression of type I IFN-induced genes, quantitative RT-PCR was performed using primers for mIRF-7 and mOasl-2 genes. Transcripts were normalized to the level of the murine β-Actin transcripts. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments in BW5147 and BaF3 cell lines. B) 12 x 10^6 BW5147 cells stably transduced by JAK1^{WT} or JAK1^{A634D} were electroporated with 15 µg of pIRF7-luc luciferase reporter plasmid, which contains the promoter of the murine IRF-7, and 1 µg of internal control pRL-TK plasmid. Cells were seeded in 96-well plates at 5 x 10^5 cells/well and stimulated with 1% IFN-β or mock/control HEK293 supernatants for 2 hours, before luciferase assay was performed. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments.*

JAK1^{A634D} potentiates type I IFNs induced cell growth inhibition. Type I IFNs are known to exert anti-proliferative activity on various cell types. Our observations suggest that JAK1^{A634D} expressing cells might be particularly sensitive to this activity. To address this hypothesis, we analyzed proliferation of cells expressing a wild-type or mutated JAK1 in the presence of IFN-β. While IFN-β reduced the proliferation of untransduced and JAK1^{WT} expressing BW5147 cells after 3 days (Figure 4A and 4C), it completely inhibited proliferation of cells expressing the JAK1^{A634D} mutant. In line with our previous observations, the effect of the A634D mutation on cell proliferation in the presence of IFN-β was dependent on receptor binding, since the expression of JAK1$^{A634D/Y107A}$ abolished cell hypersensitivity to IFN-β. Similar experiments were performed in BaF3 cells, where IFN-β treatment resulted in complete inhibition of proliferation of JAK1^{A634D} expressing cells, which was not observed in cells expressing the JAK1^{WT} or JAK1$^{A634D/Y107A}$ (Figure 4B). Interestingly, autonomous and IL-3-dependent BaF3 cells expressing JAK1^{A634D} exhibited a similar hypersensitivity to IFN-β, indicating that even cells that depend on the JAK1 mutant for their autonomous proliferation retain hypersensitivity to IFNs. Since all previous experiments were performed

using IFN-β supernatant produced by HEK293 cells, we used purified mouse recombinant IFN-β to confirm the specificity of IFN-β related inhibition of proliferation. As expected, mouse recombinant IFN-β completely inhibited proliferation of JAK1^{A634D} expressing cells, but not that of parental or JAK1^{WT} expressing BW5147 cells (Supplementary Figure 1A).

It is well known that a higher cell proliferation rate can result in a higher sensitivity to all kind of drugs. To rule out the possibility that expression of JAK1^{A634D} would induce a non-specific increase in drug sensitivty, we studied the activity of doxorubicin and cytosine arabinoside (ARA-C), two standard chemotherapy used for ALL, on the proliferation of BW5147 cells expressing JAK1^{WT} or JAK1^{A634D}. As illustrated in supplementary figures 1B and 1C, the hypersensitivity of the JAK1^{A634D} expressing cells was specific for IFN-β, since the antiproliferative activity of ARA-C or doxorubicine was not affected by the expression of JAK1^{A634D}.

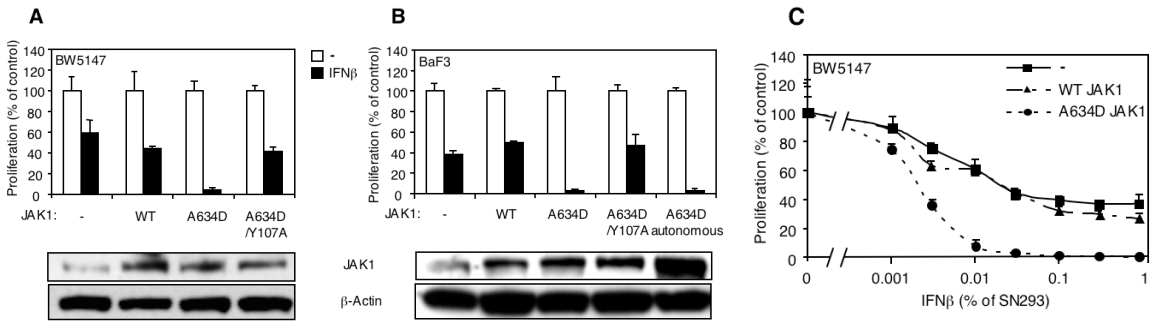

FIGURE 4 JAK1^{A634D} confers hypersensitivity to IFN-β in BW5147 and BaF3 cells. *A) 4,000 BW5147 or B) 3,000 BaF3 cells stably transduced by JAK1^{WT} or JAK1^{A634D}, with intact or mutated Y107A FERM domain (JAK1$^{A634D/Y107A}$) were seeded in 96-well plates and stimulated by 1% IFN-β or mock/control HEK293 supernatants. After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. In parallel 10^6 BW5147 or BaF3 cells were starved for 4 hours, lysed and subjected to Western Blot analysis. Total JAK1 and β-actin level was assessed using anti-JAK1 and anti-β-Actin antibodies. C) 4,000 BW5147 cells stably transduced by JAK1^{WT} or JAK1^{A634D}, with intact or mutated Y107A FERM domain (JAK1$^{A634D/Y107A}$) were seeded in 96-well plates and stimulated by IFN-β HEK293 supernatants at different dilutions, starting from 1%. After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments in BW5147 and BaF3 cell line.*

To check whether JAK1^{A634D} also confers hypersensitivity to other type I IFNs, we compared the proliferation of BW5147 cells expressing JAK1^{WT} or JAK1^{A634D} in the presence of murine IFN-α6T and IFN-α11. Previous studies on the murine B6 melanoma cell line documented that IFN-α11 displays an anti-proliferative effect which is comparable to that exerted by IFN-β, while IFN-α6T shows a lower anti-proliferative activity (19). As shown in Supplementary Figure 2, JAK1^{A634D}, but not untransduced or JAK1^{WT} expressing BW5147 cells, were hypersensitive to all tested type I IFNs.

The R724H but not the R879C mutation in JAK1 confers IFN-β hypersensitivity in BW5147 and BaF3 cells. We showed previously that, beside JAK1^{A634D}, the ALL-associated JAK1^{R724H} mutant induced autonomous growth of the cytokine-dependent BaF3 cell line, whereas a third ALL-associated mutant, JAK1^{R897C}, protected the BW5147 cell line from dexamethasone-induced apoptosis (2). These observations supported a gain of function role for these mutations. It also suggested that such a differential biological outcome might be dependent on specific effect of JAK1 mutants on signaling mediated via distinct endogenously expressed cytokine receptors. We therefore examined whether the JAK1^{R724H} and JAK1^{R879C} mutants confer hypersensitivity to IFNs in BW5147 and BaF3 cells. Remarkably, expression of the JAK1^{R724H} mutant in both cell lines slightly potentiated the inhibitory effect of IFN-β on cell proliferation, while expression of JAK1^{R879C} at similar level did not affect the IFN response in both cell lines (Figure 5). Of note, the JAK1^{R724H}-mediated inhibition of proliferation appeared to be more pronounced in BaF3 cells selected for autonomous growth. These results demonstrate a differential effect of ALL-associated *JAK1* mutations in potentiating IFNs response, with the A634D mutation having the strongest activity.

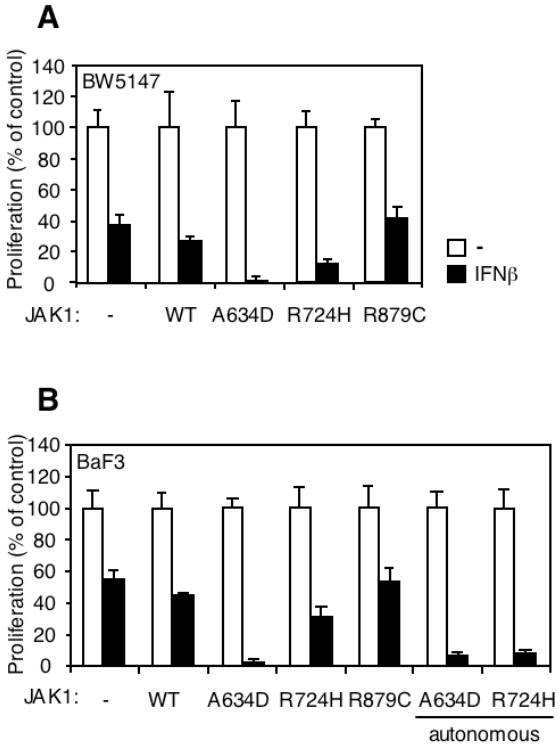

FIGURE 5 The JAK1^{R724H} and the JAK1^{A634D} mutants, but not JAK1^{R879C}, confer IFN-β hypersensitivity in BW5147 and BaF3 cells. *A) 4,000 BW5147 or B) 3,000 BaF3 cells stably transduced by JAK1 wild type or different JAK1 mutants were seeded in 96-well plates and stimulated by 1% IFN-β or mock/control HEK293 supernatants. After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments in BW5147 and BaF3 cell line.*

ALL-associated JAK1 mutants differentially potentiate IFN-β and IL-9 response. These observations support the hypothesis that different activating *JAK1* mutations might have distinct activities because they potentiate signaling by distinct cytokine receptor complexes. We took advantage of the BW5147 cell line, which endogenously express two JAK1-binding receptors (IL-9Rα and IFNAR2) to compare the activity of different JAK1 mutants on distinct signaling pathways. The STAT3-responsive pGL3-pap1-luc reporter construct and quantitative RT-PCR for the STAT3-induced Bcl-3 gene were used as read-out for the IL-9 receptor signaling pathway (20,21). For the type I IFNs pathway, we used the ISGF3 pIRF7-luc reporter plasmid and a quantitative RT-PCR for IRF-7. As shown in Figure 6, the effect of IL-9 was increased by the three JAK1 mutants, as documented by luciferase assay and Bcl-3 expression. By contrast, only JAK1^{A634D} significantly increased IFN response, in line with the proliferation assays described above. Taken together, our observations suggest that ALL-associated JAK1 mutants have overlapping but also distinct intrinsic specificity to dysregulate signaling elicited by cytokine receptors, explaining the observed differential cytokine hypersensitivity.

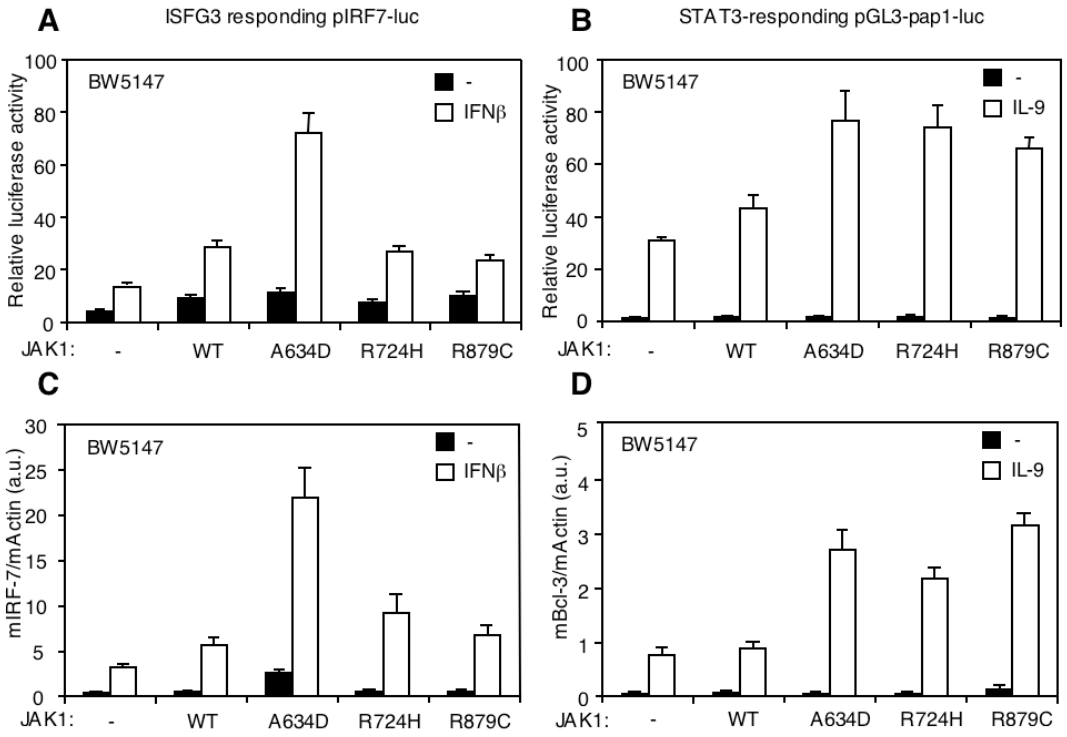

FIGURE 6 JAK1^{A634D}, JAK1^{R724H} and JAK1^{R879C} differentially favor IFN-β and IL-9 signaling. *A) and B) 12 x 10^6 BW5147 cells stably transduced by JAK1^{WT} or JAK1^{A634D} were electroporeted with 15 µg of the ISGF-3 responsive pIRF7-luc (A) or the STAT3 responsive pGL3-pap1-luc (B) luciferase reporter plasmid and 1 µg of internal control pRL-TK plasmid. Cells were seeded in 96-well plates at 5 x 10^5 cells/well and stimulated with 1% IFN-β or mock/control HEK293 supernatants (for pIRF7-luc) and by 100U/ml hIL-9 (for pGL3-pap1) for 2 hours, before luciferase assay was performed. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments. C) and D) 10^6 BW5147 cells stably transduced by JAK1 wild-type (WT) or different JAK1 mutants were stimulated with 1% IFN-β or mock/control HEK293 supernatants (C) and stimulated with 100 U/ml of hIL-9 (D) for 3 hours, lysed and used for total RNA extraction. Real-time PCR was performed to quantify transcripts of mIRF-7 and mBcl-3 genes. Gene expressions were normalized to the level of the murine β-Actin transcripts. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments.*

JAK1^{A634D}expressing leukemic cells are more sensitive to the anti-tumorigenic effect of type I IFN in vivo. Type I interferon are used in the clinic to treat several malignancies. Our *in vitro* data suggest that leukemic cells harboring a mutation in *JAK1* are more sensitive to the anti-proliferative effect of type I IFN. To check the relevance of this observation *in vivo*, we tested the effect of IFN-α in a murine model of leukemia, based on the intraperitoneal injection of the murine T cell leukemia BW5147 cell line in immunocompromised mice. Treatment with IFN-α was achieved by intramuscular electroinjection of a murine IFN-a construct, a procedure that generates endogenous production of IFN-α with systemic effects lasting for 3 to 4 months (11).

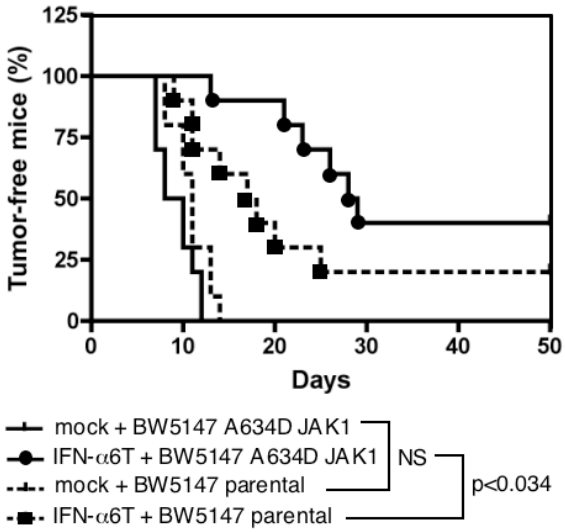

FIGURE 7 JAK1^{A634D} potentiates the anti-tumoral effect of type I IFN in vivo*. Rag2-/- mice received an intramuscular injection of an IFN-α6T construct or of an empty vector. Two days later, 10^6 BW5147 leukemia cells expressing or not JAK1^{A634D} were injected i.p. (10 mice/group). Kaplan-Meier curves of tumor development are shown. A Gehan-Breslow-Wilcoxon test shows that JAK1^{A634D} expression significantly delayed tumor formation compared to control cells (p<0.034). These results were reproduced in 3 experiments with either Rag2 -/- or SCID mice.*

As shown in Figure 7, control mice receiving BW5147 cells showed tumor development after 1 week, and all mice developed tumor after 2 weeks. This was similar for mice receiving JAK1^{A634D} expressing BW5147 cells. Treatment with IFN-α delayed the tumors formation of BW5147 cells, but this effect was more pronounced for JAK1^{A634D} expressing BW5147 cells. Thus, compared to control cells, JAK1^{A634D} expressing cells were significantly (p=0.034) more sensitive to the anti-tumorigenic effect of IFN-α *in vivo*.

DISCUSSION

In this paper, we made three important observations. First, we identified a type I IFN transcriptional signature in human ALL cells harboring activating mutations in *JAK1*. This signature can be explained by the constitutive activation of the IFN receptor-signaling complex by the mutated JAK1 protein. Secondly, we showed that different JAK1 mutants activate distinct cytokine-receptor signaling complexes, demonstrating specificity in signaling pathways activation based on the type of mutation in JAK1. Finally, we demonstrated that the JAK1^{A634D} mutant confers hypersensitivity to the anti-tumorigenic effect of type I IFN *in vivo*, suggesting that IFN-a can be used as adjuvant treatment for *JAK1* mutation-positive malignancies.

Recently, we and others reported that acquired gain of function mutations in *JAK1* contribute to leukemogenesis (2-4). These mutations appear to represent a relatively common event in adult T-cell ALL, while they are more rare in adult B-cell ALL and in childhood malignancies (2,4). We also showed that JAK1 mutants need to associate to cytokine receptors, such as IL-2R and IL-9R, to promote constitutive activation of the receptor

complex and signal transduction via STAT3 and STAT5 (5). In this model, the receptor has a dual role to play: it serves as a scaffold for cross-activation of JAKs and as a docking site for STATs. Activation of different STAT transcription factors and aberrant expression of the related STAT-regulated genes might therefore reflect the activation of distinct cytokine receptor complexes. In this work, we observed that human *JAK1* mutation-positive T-ALL samples were characterized by an overexpression of type I IFN stimulated genes (ISGs), suggesting constitutive activation of the type I IFN signaling pathway. This observation is best explained by the activation of the type I IFN receptor complex by the activated JAK1 protein. Several arguments favor this hypothesis: (i) IFNAR2, which is one of the chain of the type-I IFN receptor, binds JAK1; (ii) the Y107A mutation, which abolishes JAK1 capacity to bind to receptors also abolishes the constitutive activation of the IFN pathway; (iii) TYK2, the kinase associated to IFNAR1, which is the other chain of the receptor, is constitutively activated in cell lines expressing the mutated form of JAK1, suggesting cross-activation of TYK2 by hyperactive JAK1; (iv) STAT2, which is specifically activated through the type I IFN receptor complex, is also constitutively activated in cell lines expressing the ALL-associated mutated JAK1 allele.

The other JAK required for type I IFN signaling is TYK2, which is appended to IFNAR1. An activating mutation in TYK2 (V678F, homologous to $JAK2^{V617F}$) (22), constitutively activated STAT3, but not the canonical IFN-signaling pathway, and did not lead to activation of JAK1 (18). Furthermore, in the model of 11.1 mutant cells that lack TYK2, mutant TYK2 did not affect IFN-induced signaling (18). By contrast, in BW5147 and BaF3 cells, expression of JAK1 mutants, namely A634D and R724H, potentiated the activation of type I IFN receptor signal transduction by IFN. Most importantly, the increased ligand-responsiveness resulted into the hypersensitivity of $JAK1^{A634D}$ mutant-expressing cells to type I IFN mediated anti-proliferative effect *in vitro* and in almost complete inhibition of proliferation of these cells by several type I IFN ligands.

The fact that $JAK1^{A634D}$ mutant-expressing tumor cells, which are hypersensitive to type 1 IFN, express a constitutive IFN signature, but yet proliferate *in vivo* might seem paradoxical. However, it must be stressed that the IFN signature that we observe in $JAK1^{A634D}$ mutant-expressing cells remains below the gene expression level reached upon IFNß stimulation, even if JAK1 is not mutated, as illustrated in figure 3. Thus, JAK1 mutation results in a low level of IFN signaling, below the antiproliferative threshold, and renders cells hyperresponsive to further IFN stimulation.

Among the three functionally characterized ALL-associated JAK1 mutants, JAK^{A634D} was observed to be the strongest potentiator of the IFN response, while $JAK1^{R724H}$ weakly potentiate cell response to IFN and $JAK1^{R879C}$ had no effect. However, in the same cell line endogenously expressing IL-9R, all three JAK1 mutants conferred increased IL-9 responsiveness to a similar extend. We also previously showed that, beside the A634D mutant, the R724H JAK1 mutant induced autonomous growth of the IL-3-dependent BaF3 cell line, whereas the R897C mutant did not. In contrast, this JAK1 R897C protected the BW5147 cell line from dexamethasone-induced apoptosis (2). These findings are best explained by the idea that the capability of each JAK1 mutant to promote intracellular signaling is strictly dependent upon the receptor-dependent context, and by the differential expression of a set of cytokine receptors. Altogether these data document a level of specificity based on the type of mutation in JAK1 and strongly suggest that the different ALL-associated JAK1 mutants do not have a homogeneous dysregulating effect on intracellular signaling. This specificity could be explained by either a mutation-specific conformational change of the kinase resulting in a different binding affinity for the diverse cytokine receptors, a different capacity to cross-activate other JAK bound to the second chain of the receptor complex, or a difference in substrate specificity. Of note, this hypothesis could explain why the V617F mutation in JAK2 is so frequent in myeloproliferative neoplasms. Based on the homology of JAK1 and JAK2, we can predict that mutations in JAK2 that are homologous to the mutations in JAK1 will activate JAK2. However, the V617F mutation is nearly the only one observed in patients. This mutation might specifically potentiate signaling pathways that would not be activated by other mutants, thereby representing the 'best hit' to mediate myeloproliferation.

Type I interferons are used in the clinic as adjuvant to treat patients with renal cell carcinoma and melanoma (13). The way interferon exerts its anti-tumoral effect in these diseases is not perfectly understood, but it is considered as an immunotherapy that potentiate the immune response against the tumor. The treatment with interferon is safe and induces few side-effects compared to chemotherapies that are usually used in these malignancies. The pegylated form of interferon allows for less frequent injection (once weekly) and easier administration. Interferon-α is also used to treat patients with myeloproliferative neoplasms, such as essential thrombocythemia or polycythemia vera. In these diseases, interferon is thought to exert an anti-proliferative effect. Recent data suggest that interferon is the only medication able to induce molecular remission in these diseases, as shown by the disappearance of the JAK2 V617F mutation in a substantial number of patients (14). Interferon is not used to treat ALL. Our data show that different JAK1 mutants specifically

potentiate the anti-proliferative effect of interferon. Moreover, our *in vivo* leukemia model shows that treatment with interferon decreases tumor formation of cells expressing JAK^{A634D}. These data suggest that interferon-α might represent a candidate as adjuvant targeted therapy for ALL patients harboring *JAK1* mutations, which are present in up to 18 % of adult T-ALL subjects (2).

MATERIALS AND METHODS

Patients and microarray data analyses. Cohorts used for gene expression profiling included 3 *JAK1* mutation-positive (S512L, R724H 2x) and 8 mutation-negative T-ALL samples from patients enrolled in the adult clinical trial Gruppo Italiano Malattie Ematologiche dell'Adulto 0496 and 2000 as described previously (2). 6 *JAK1* mutation-positive (S512L, R724H/C/Q and Y652H 2x) and 11 mutation-negative T-ALL samples from the GRAALL (Group for Research on Adult Acute Lymphoblastic Leukemia) (16) were used as an independent cohort to confirm our gene expression profiling data. Written informed consent for genetic analyses was obtained from all subjects according to the Declaration of Helsinki. Raw Affymetrix microarray data were obtained as described previously (2) and are accessible at GEO public database (GSE18239). Briefly, RNA was extracted from 3 adult T-ALL samples harboring a mutation in *JAK1* (S512L, 2 samples R724H) and 8 T-ALL samples with wild-type *JAK1*. These samples contained more than 90% of ALL blasts. RNA was labeled with the standard Affymetrix procedure and hybridized to the HG-U133 Plus 2.0 arrays. Raw data were normalized by global scaling on all probe sets, with a target signal of 100. Genes called absent in more than 8 samples were removed from the analysis, which was performed on the remaining 27,049 genes. For GSEA, a supervised comparison of the *JAK1* mutation-positive and -negative blast samples was performed with the Significance Analysis of Microarrays (SAM) tool implemented in the TIGR MeV software (http://www.tm4.org/mev.html). Genes were classified from 1 (more expressed in the JAK1 mutated samples) to 27,049 (genes more expressed in the JAK1 wild-type samples) according to the SAM score. A preranked analysis was performed in the GSEA software (http://www.broad.mit.edu/cancer/software/gsea) using the curated genesets (23). By this analysis, 20 genesets were significantly (FWER p-val < 0.05) enriched in the *JAK1* mutation-positive blast samples, and 20 genesets were significantly enriched in the *JAK1* mutation-negative blast samples. The clustering based on an independent list of IFN stimulated genes (ISGs) was performed as follow: from the 39 genes induced by IFN in PBMC (15), 29 genes were called present in more than 3 ALL samples. These genes were represented by 80 probe sets. The analysis was performed based

on these probe sets. A hierarchical clustering of the samples and of the genes, based on the Euclidian distance, was performed in the TIGR MeV software.

Cell culture and cytokines. BaF3 mouse hematopoietic pro-B cells were cultured in Dulbecco's modified Eagle's medium with fetal bovine serum (10%) and IL-3 (100 U/ml). BW5147 mouse T-cell leukemia cells (24) were grown in Iscove-Dulbecco's medium supplemented with 10% fetal bovine serum, 0.55 mM L-arginine, 0.24 mM L-asparagine, and 1.25 mM L-glutamine. Recombinant human IL-9 was produced in the baculovirus system and purified in our laboratory. Murine IFN-α6T, -α11 and -β supernatants were produced by transient transfection of HEK293 cells as described previously (19). Control supernatant was produced using the corresponding empty vector. Typically, HEK293 cells produced supernatants contained 250.000 U/ml of IFNs (19). Recombinant murine IFN-β was purchased from PBL Interferon Source.

Plasmid construction, stable DNA transfections and analysis of transfected cells. All JAK1 mutants (A634D, A634D/Y107A, R724H, R879C) were generated using QuickChange XL II site-directed mutagenesis kit (Stratagene) and subcloned into the pMX-GFP biscistronic retroviral vector upstream of the IRES as previously described (2,5,22). For stable transduction, 0.5 x 10^6 BW5147 or BaF3 cells were infected by retroviruses produced by BOSC packaging cells using standardized protocol previously described (25). Cells were subsequently sorted by FACS for equal level of GFP.

Transient Cell Transfection and Luciferase assay. Two different reporter plasmids were used: pGL3-pap1-luc and pIRF7-luc. STAT3-mediated transcription was evaluated with pGL3-pap1-luc plasmid (obtained from Dr. Jan Tavernier, University of Ghent, Belgium) containing the luciferase gene under the control of the STAT3-inducible rat *Pap1* promoter (26). The pIRF7-luc reporter plasmid contains promoter of the murine IRF-7 (21), which is able to bind ISGF3 complex. Another plasmid, pRL-TK (Promega), encoding Renilla luciferase, was co-transfected as an internal control of the electroporation process. Briefly, 12 x 10^6 BW5147 cells stably transduced with the different JAK1 constructs were electroporeted with 15 μg of luciferase reporter plasmid and 1 μg of internal control pRL-TK plasmid, and were seeded in 96-well plates at 5 x 10^5 cells/well. Cells were stimulated by mock/control or IFN-β HEK293 supernatants or hIL-9 (100 U/ml) for 2 hours before luciferase assays were performed using the dual luciferase reporter kit (Promega).

Western blots. For Western Blot analysis, 10^6 BW5147 or BaF3 stably transduced cells were starved for 4 hours, then collected and lysed in 250 µl of Laemmli buffer (BioRad) using a 20-gauge syringe. Phosphorylation of signaling proteins was investigated by Western blot as described previously (5) using following phospho-specific antibodies from Cell Signaling Technology: anti-pY1022/1023 JAK1 (#3331S), anti-pY1054/1055 TYK2 (#9321S), anti-pY701 STAT1 (#9171S) and from Upstate anti-pY689 STAT2 (#07-224). Blots were re-probed with anti-JAK1 (#3332), anti-STAT2 (#4597) (Cell Signaling Technology), anti-STAT1 (#S21120) (Signal Transduction Laboratory) or anti-β-Actin (Sigma) antibodies, as a control.

Proliferation assay. 4,000 BW5147 or 3,000 BaF3 stably transduced cells were seeded in 96-well plates and stimulated by murine IFN-α6T, -α11, -β and mock/control HEK293 supernatants or recombinant murine IFN-β at different dilutions, starting from 1%. Alternatively, cells were treated by various concentrations of doxorubicin (Sigma) or cytosine arabinoside (ARA-C) (Sigma). For all conditions, 150 U/ml of IL-3 was added to the BaF3 cell cultures to ensure proliferation of the non-autonomous cells. After 72 hours, tritiated thymidine was added to the cells for 4 hours, and thymidine incorporation was measured with a Top Count microplate scintillation counter (Canberra-Packard).

Reverse transcription-quantitative PCR (RT-PCR). 10^6 cells were stimulated for 3 hours with IL-9 (100 U/ml) or with IFN-β (1% HEK293 SN), or left unstimulated. Total RNA was extracted using the TriPure isolation reagent (Roche) according to the manufacturer's instructions. Reverse transcription was performed on 1 µg of total RNA with an oligo-(dT) primer (Roche) and M-MLV RT (Invitrogen). Quantitative PCR reactions were performed using 50 µl cDNA and primer sets corresponding to murine IRF-7, murine Oasl-2, murine Bcl-3 and murine β-Actin with qPCR™ Mastermix for SYBR Green I (Eurogentec). The sequences of murine primers (final concentration: 300 nM) were: mIRF-7: 5'-CAGCGAGTGCTGTTTGGAGAC-3' (forward) and 5'-AAGTTCGTACACCTTATGCGG-3' (reverse); mOasl-2: 5'-GGATGCCTGGGAGAGAATCG-3' (forward) and 5'-TCGCCTGCTCTTCGAAACTG-3' (reverse); mBcl-3, 5'-TCCTGAGCGCAAGTACTCTGT-3' (forward), 5'-CTGATCCACATCTGCTGGAAG-3'(reverse); mβ-Actin 5'-GCTGGAAGGTGGACAGTGAG-3' (forward), 5'-

CTCTGGCTCCTAGCACCATGAAG-3' (reverse). The sequences of human primers (final concentration: 300 nM) were: hIRF-7 5'-AGAAGAGCCTGGTCCTGGTGAA-3' (forward), 5'-CGATGTCGTCATAGAGGCTGTTG-3' (reverse); hMX1 5'-GAGCTGCCAGGCTTTGTGAA-3' (forward), 5'-GGCGGATCAGCTTCTCACCT-3' (reverse); hβ-Actin 5'-GTCATACTCCTGCTTGCTGA-3' (forward), 5'-ATTGCCGACAGGATGCAGAA-3' (reverse) and 5'-TCAAGATCATTGCTCCTCCTGAGC-3' (probe). Samples were first heated 2 min at 50°C then 10 min at 95°C. cDNA was amplified as follows: 40 cycles of a two-step PCR program at 95°C for 15 sec and 60°C for 1 min. Melting point analysis was carried out by heating the amplicon from 60°C to 95°C. Results were analyzed by MyiQ software. Starting quantity of the transcripts was calculated using standard curves from a cDNA clone. Expression of all genes was normalized to the expression of β-Actin.

Mice. Female 6- to 9-week-old SCID or RAG2-/- mice were obtained from the animal facility of the Université catholique de Louvain, Belgium. Handling of mice and experimental procedures were conducted in accordance with national and institutional guidelines for animal care.

In vivo leukemia model. BW5147 is a T leukemia cell line obtained from AKR mice (24). BW5147 cells or BW5147 cells expressing $JAK1^{A634D}$ were washed and resuspended at the concentration of 10^6 cells in 200 µl of PBS. 10^6 cells were injected intraperitoneally to mice 2 days after the intramuscular electroinjection of murine IFN-α6T subcloned into pcDNA3 or the empty vector. Mice were kept in sterile cages with sterile water and food ad libitum. Mice were examined daily for 3 months in a blind way for tumor growth. Results were represented by Kaplan-Meier curves and statistics were performed using the Gehan-Breslow-Wilcoxon test in the package "survival" of the program R (http://www.R-project.org.).

DNA electroinjection. Mice were anesthetized with 200 µl of a mix of Medetomidin hydrochlorid 100 µg/ml (Dormitor) and Ketamine 500 µg/ml (Anesketin) given i.m. Before DNA injection, mice were shaved locally, using depilatory cream. 10 µg of endotoxin-free plasmid DNA (Promega endofree) in 25 µl of PBS were injected in the left and right tibialis anterior muscles of the mice. Electric pulses (80 V per 4 mm, 8 pulses, 20 msec/pulse, pause: 480 msec) were administered using a Cliniporator system (Cliniporator, IGEA, Capri, Italy) equipped with 6 mm electrode plates. For all experiments, conductive gel was used to ensure

electrical contact with the skin (EKO-GEL, ultrasound transmission gel, Egna, Italy). Mice were woken up by i.m. injection of 250 µl of Atipamezol 500 µg/ml (Antisedan).

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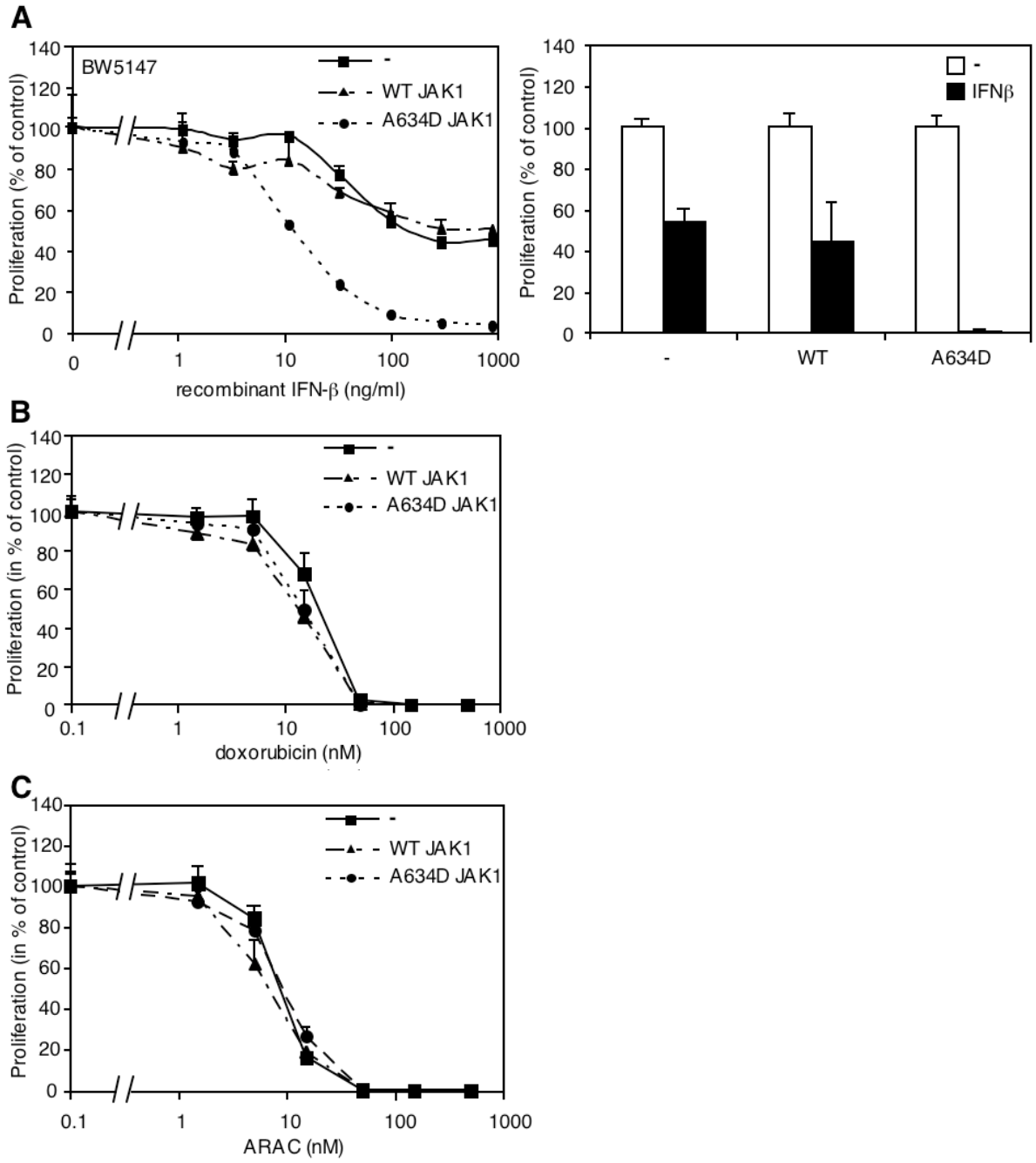
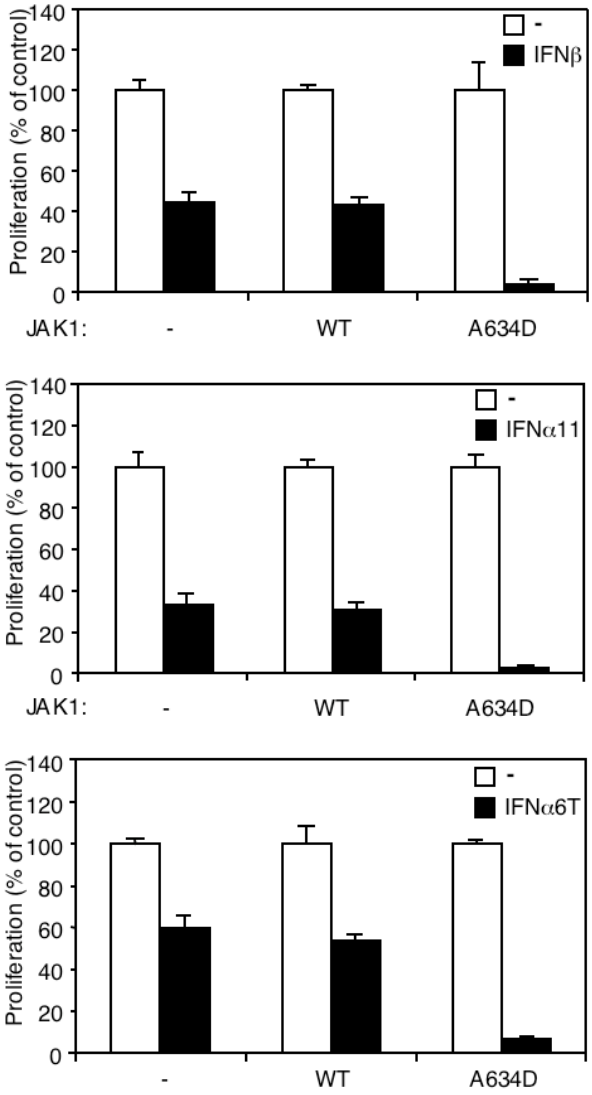
REFERENCES

1. Schindler, C., Levy, D. E., and Decker, T. (2007) *J Biol Chem* **282**, 20059-20063
2. Flex, E., Petrangeli, V., Stella, L., Chiaretti, S., Hornakova, T., Knoops, L., Ariola, C., Fodale, V., Clappier, E., Paoloni, F., Martinelli, S., Fragale, A., Sanchez, M., Tavolaro, S., Messina, M., Cazzaniga, G., Camera, A., Pizzolo, G., Tornesello, A., Vignetti, M., Battistini, A., Cave, H., Gelb, B. D., Renauld, J. C., Biondi, A., Constantinescu, S. N., Foa, R., and Tartaglia, M. (2008) *J Exp Med* **205**, 751-758
3. Jeong, E. G., Kim, M. S., Nam, H. K., Min, C. K., Lee, S., Chung, Y. J., Yoo, N. J., and Lee, S. H. (2008) *Clin Cancer Res* **14**, 3716-3721
4. Mullighan, C. G., Zhang, J., Harvey, R. C., Collins-Underwood, J. R., Schulman, B. A., Phillips, L. A., Tasian, S. K., Loh, M. L., Su, X., Liu, W., Devidas, M., Atlas, S. R., Chen, I. M., Clifford, R. J., Gerhard, D. S., Carroll, W. L., Reaman, G. H., Smith, M., Downing, J. R., Hunger, S. P., and Willman, C. L. (2009) *Proc Natl Acad Sci U S A* **106**, 9414-9418
5. Hornakova, T., Staerk, J., Royer, Y., Flex, E., Tartaglia, M., Constantinescu, S. N., Knoops, L., and Renauld, J. C. (2009) *J Biol Chem* **284**, 6773-6781
6. Lu, X., Levine, R., Tong, W., Wernig, G., Pikman, Y., Zarnegar, S., Gilliland, D. G., and Lodish, H. (2005) *Proc Natl Acad Sci U S A* **102**, 18962-18967
7. Lu, X., Huang, L. J., and Lodish, H. F. (2008) *J Biol Chem* **283**, 5258-5266
8. Uze, G., Schreiber, G., Piehler, J., and Pellegrini, S. (2007) *Curr Top Microbiol Immunol* **316**, 71-95
9. Taniguchi, T., and Takaoka, A. (2001) *Nat Rev Mol Cell Biol* **2**, 378-386
10. Renauld, J. C. (2003) *Nat Rev Immunol* **3**, 667-676
11. Sommereyns, C., Paul, S., Staeheli, P., and Michiels, T. (2008) *PLoS Pathog* **4**, e1000017
12. Chawla-Sarkar, M., Lindner, D. J., Liu, Y. F., Williams, B. R., Sen, G. C., Silverman, R. H., and Borden, E. C. (2003) *Apoptosis* **8**, 237-249
13. Jonasch, E., and Haluska, F. G. (2001) *Oncologist* **6**, 34-55
14. Kiladjian, J. J., Chomienne, C., and Fenaux, P. (2008) *Leukemia* **22**, 1990-1998
15. Rani, M. R., Shrock, J., Appachi, S., Rudick, R. A., Williams, B. R., and Ransohoff, R. M. (2007) *J Leukoc Biol* **82**, 1353-1360
16. Asnafi, V., Le Noir, S., Lhermitte, L., Gardin, C., Legrand, F., Vallantin, X., Malfuson, J. V., Ifrah, N., Dombret, H., and Macintyre, E. (2009) *Br J Haematol*
17. Lehtonen, A., Matikainen, S., and Julkunen, I. (1997) *J Immunol* **159**, 794-803
18. Gakovic, M., Ragimbeau, J., Francois, V., Constantinescu, S. N., and Pellegrini, S. (2008) *J Biol Chem* **283**, 18522-18529
19. van Pesch, V., Lanaya, H., Renauld, J. C., and Michiels, T. (2004) *J Virol* **78**, 8219-8228
20. Richard, M., Louahed, J., Demoulin, J. B., and Renauld, J. C. (1999) *Blood* **93**, 4318-4327

21. Dumoutier, L., Tounsi, A., Michiels, T., Sommereyns, C., Kotenko, S. V., and Renauld, J. C. (2004) *J Biol Chem* **279**, 32269-32274
22. Staerk, J., Kallin, A., Demoulin, J. B., Vainchenker, W., and Constantinescu, S. N. (2005) *J Biol Chem* **280**, 41893-41899
23. Subramanian, A., Tamayo, P., Mootha, V. K., Mukherjee, S., Ebert, B. L., Gillette, M. A., Paulovich, A., Pomeroy, S. L., Golub, T. R., Lander, E. S., and Mesirov, J. P. (2005) *Proc Natl Acad Sci U S A* **102**, 15545-15550
24. Fekete, E., and Kent, E. (1955) *Transplant. Bull.* **2**, 61
25. Seubert, N., Royer, Y., Staerk, J., Kubatzky, K. F., Moucadel, V., Krishnakumar, S., Smith, S. O., and Constantinescu, S. N. (2003) *Mol Cell* **12**, 1239-1250
26. Peelman, F., Iserentant, H., De Smet, A. S., Vandekerckhove, J., Zabeau, L., and Tavernier, J. (2006) *J Biol Chem* **281**, 15496-15504



SUPPLEMENTARY FIGURE 1 JAK1^{A634D} specifically confers hypersensitivity to type I IFNs. *4,000 BW5147 cells parental and stably transduced by JAK1^{WT} or JAK1^{A634D} were seeded in 96-well plates and stimulated (or left unstimulated) by different dose of (A) murine recombinant IFN-β, (B) doxorubicin and (C) cytosine arabinoside (ARA-C). After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments.*



SUPPLEMENTARY FIGURE 2 JAK1^{A634D} confers hypersentitivity to IFN-β, IFN-α11 and IFN-α6T. *4,000 BW5147 cells parental and stably transduced by JAK1^{WT} or JAK1^{A634D} were seeded in 96-well plates and stimulated by 1% IFN-β, -α11, -α6T or mock/control HEK293 supernatants. After 72 hours, tritiated thymidine was added to the cells for 4 hours and thymidine incorporation was measured. Results are mean ± SD of biological triplicates. Similar results were obtained in 3 independent experiments in BW5147 and BaF3 cell lines.*