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Deregulated JAK/STAT signalling in lymphomagenesis, and its implications for the development of new targeted therapies

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Abstract

Gene expression profiling has implicated several intracellular signalling cascades, including the JAK/STAT pathway, in the pathogenesis of particular subtypes of lymphoma. In marked contrast to the situation in patients with either acute lymphoblastic leukaemia or a myelo-proliferative neoplasm, JAK2 coding sequence mutations are rare in lymphoma patients with an activated JAK/STAT “signature”. This is instead the consequence of mutational events that result in the increased expression of non-mutated JAK2; positively or negatively affect the activity of other components of the JAK/STAT pathway; or establish an autocrine signalling loop that drives JAK-mediated cytokine-independent proliferation. Here, we detail these genetic lesions, their functional consequences, and impact on patient outcome. In light of the approval of a JAK1/JAK2 inhibitor for the treatment of myelofibrosis, and preliminary studies evaluating the efficacy of other JAK inhibitors, the therapeutic potential of compounds that target JAK/STAT signalling in the treatment of patients with lymphoma is also discussed.

Keywords: JAK2, STAT signalling, lymphoma, JAK inhibitors.
**Introduction**

The lymphomas are lymphoid malignancies that arise after the transformation of maturing B- or T-lymphocytes. There are over 30 distinct clinical entities that fall within this disease classification, with the most frequently occurring being follicular lymphoma (FL), diffuse large B-cell lymphoma (DLBCL) and Hodgkin’s lymphoma (HL). It is estimated that, in 2011 in the United States alone, more than 700,000 people were living with lymphoma and another 80,000 individuals being diagnosed each year (Surveillance, Epidemiology, and End Results (SEER) Program; [http://seer.cancer.gov/statfacts/](http://seer.cancer.gov/statfacts/)).

The diversity of diseases categorised as a lymphoma reflects both the developmental stage of the transformed lymphocyte, and the molecular events that underlie its transformation. In the last several years, use of next-generation sequencing technologies to interrogate the genome and transcriptome of various lymphoma subtypes has provided significant insights into their molecular aetiology. Amongst the recurrent somatic lesions identified in this manner are NOTCH2 truncating mutations, which occur in patients with splenic marginal zone lymphoma\(^1\); missense ID3 mutations in those with Burkitt’s lymphoma (BL)\(^3\); inactivating CREBBP mutations in cases of follicular lymphoma (FL) or diffuse large B-cell lymphoma (DLBCL)\(^4\); loss-of-function mutations in MLL2 in DLBCL\(^5\); \(^6\); and inactivating RHOA mutations in angioimmunoblastic T-cell lymphoma.\(^7\) Prior to this wave of mutation discovery, advances in gene expression profiling had already implicated several well-characterized intracellular signalling pathways in the pathogenesis of one or more lymphoma subtypes; these include the NF-κB and JAK/STAT signalling pathways. For example, the molecular signatures associated with primary mediastinal B-cell lymphoma (PMBL) and Hodgkin’s lymphoma (HL) are characterized in part by the over-expression of genes that encode constituents in the JAK/STAT signalling pathway, such as the receptor for interleukin (IL) 13, and JAK2 and STAT1 themselves.\(^41,42\) It is hoped that the identification
of mutations that are pivotal to the initiation of lymphomagenesis, through genomic or transcriptomic profiling, will translate into the development and implementation of novel targeted therapies for the treatment of lymphoma.

In this review article, we outline the mutations that are present in various subsets of patients with lymphoma and deregulate JAK/STAT signalling, and discuss their functional consequences and impact on disease outcome. In light of pre-clinical studies evaluating the efficacy of JAK inhibitors in patients with lymphoma and the approval of one such drug, Ruxolitinib, for the treatment of patients with myelofibrosis, we furthermore summarize the therapeutic potential of several compounds that specifically inhibit JAK/STAT signalling.

The basics of the JAK/STAT intracellular signalling pathway

In vertebrates, the Janus kinase (JAK) family of cytoplasmic tyrosine kinases is comprised of four closely related members: JAK1, JAK2, JAK3 and tyrosine kinase 2 (TYK2). Each JAK protein is constitutively associated with a cytokine receptor that itself lacks intrinsic tyrosine kinase activity. Within the hematopoietic system, these include the receptors for granulocyte colony-stimulating factor, erythropoietin, thrombopoietin and numerous interleukins. Receptors use different JAK protein combinations for intracellular signal transduction: for example, the erythropoietin receptor utilizes JAK2 exclusively, whereas the thrombopoietin receptor uses JAK1 and JAK2. In contrast, the IL receptors activate JAK1 or JAK2 via their ligand-specific α chain, and JAK3 via their common γ chain.

The JAK proteins each contain four distinct domains (Figure 1A). The FERM (“band 4.1, ezrin, radixin and moesin”) domain mediates their interactions with cytokine receptor subunits, whereas the Src homology-2 (SH2) domain mediates interactions with positive or negative regulators of JAK kinase activity. The JAK proteins also contain two domains with significant homology to tyrosine kinase domains: these are referred to as the JAK-homology-
1 and JAK-homology-2 (JH1 and JH2) domains. However, JH2 domains lack several features traditionally considered important for a functioning kinase. Nonetheless, the JH2 domain of JAK2 has an essential role in suppressing basal kinase activity,\textsuperscript{8,9} which might explain why it is the predominant target for acquired activating mutations associated with a hematologic malignancy. In a cytokine-free environment, this domain is constitutively phosphorylated on serine-523 and tyrosine-570, which strengthens inhibitory interactions between it and the JH1 domain, thereby suppressing the kinase activity of the JH1 domain.\textsuperscript{10,11} Engagement of a receptor with its cognate ligand induces structural changes within the receptor,\textsuperscript{12} facilitating JAK2 auto-phosphorylation on tyrosine residues Y1007 and Y1008, with a concomitant decrease in the levels of phosphorylated serine-523 and tyrosine-570. Upon activation, the JAK proteins phosphorylate tyrosine residues within the cytoplasmic domain of the receptors to which they are bound, thereby providing docking sites for various signalling proteins, such as members of the signal-transducer-and-activator-of-transcription transcription factor family (STATs 1, 2, 3, 4, 5A, 5B and 6). Recruited STAT monomers become activated by JAK-mediated phosphorylation, dimerise and translocate into the nucleus, where they enhance transcription at specific loci. Activated JAKs also induce the activation of other downstream signalling cascades, including the MAP kinase and PI-3-kinase/AKT pathways.

It has long been appreciated that the JAK proteins are not exclusively cytoplasmic, but little was known about their nuclear activity. In 2006, studies of modifiers of JAK activity in \textit{Drosophila} identified a non-canonical JAK signalling pathway in which activated Hopscotch (Hop), the single JAK protein in flies, disrupts gene silencing by displacing heterochromatin protein-1 (Hp1), which in turn induces the aberrant expression of normally silenced genes.\textsuperscript{13} Deregulated expression of several of these genes led to the development of a leukaemia-like phenotype in flies that carry an activating Hop mutation. Nuclear JAK2 is also present in mammalian hematopoietic cell-lines and primary CD34\textsuperscript+ progenitors, but not
in mature blood cells.\textsuperscript{14, 15} There, JAK2 directly phosphorylates histone H3 on tyrosine-41 (H3Y41), which excludes the chromo-shadow domain of the heterochromatin protein, HP1\(\alpha\), from binding to this site. HP1\(\alpha\) displacement leads to alterations in the chromatin structure that surrounds transcriptionally inactive genes.\textsuperscript{15, 16} Accordingly, phosphorylated H3Y41 is localized to a subset of active genomic promoters, and present throughout the coding region of functionally important genes such as \textit{GATA2} and \textit{TAL1}.\textsuperscript{17} Phospho-H3Y41 is also present at \textit{cis} regulatory elements to which STAT5 is bound, demonstrating that the transcription of some loci is regulated by both the canonical and non-canonical JAK signalling pathways.

Histone phosphorylation is not the only effect that JAK2 has on chromatin structure. It also interacts with PMRT5, an arginine methyltransferase that mediates the di-methylation of arginine residues within the H2A, H3 and H4 histones.\textsuperscript{18, 19} JAK2-mediated phosphorylation of PMRT5 significantly reduces its methyltransferase activity, changing the pattern of histone modification within the cell, and altering gene expression patterns.\textsuperscript{19} JAK2 has also recently been shown to phosphorylate EZH2,\textsuperscript{20} a methyltransferase that is the catalytic subunit of the polycomb repressive complex 2 (PRC2). Unmodified EZH2 inhibits gene transcription by methylating histone H3 on lysine-27 (H3K27)\textsuperscript{21, 22}; phosphorylation targets it for proteosomal degradation, thereby alleviating this transcriptional repression.

As discussed in a later section of this review, genomic amplification that results in the over-expression of non-mutated JAK2 in some lymphoma sub-types has an impact on both canonical and non-canonical JAK signalling pathways in mutation-positive cells.

\textit{JAK3, but not JAK2, point mutations are associated with lymphomagenesis}

Given that a significant proportion of patients with lymphoma display abnormal JAK/STAT activation, it is reasonable to question whether this may be the result of mutations that affect one or more members of the JAK family members. Mutations in JAK1 and TYK2 have not
been observed in lymphoma patients, although JAK2 and JAK3 mutations have been detected in a proportion of cases. Somatic JAK2 mutations occur frequently in some hematologic malignancies, particularly those affecting the myeloid lineages. An acquired activating mutation, JAK2V617F, is present in the majority of patients diagnosed with a myeloproliferative neoplasm (MPN), whereas JAK2 “exon 12” mutations are acquired by a subset of patients that lack the JAK2V617F mutation (Figure 1A). However, JAK mutations are not only associated with the transformation of myeloid cells; a third type of JAK2 activating mutation, which affects arginine-683 or adjacent residues, is present in 20% of patients with Down syndrome-associated or high-risk sporadic acute lymphoblastic leukaemia (ALL). In contrast, JAK2 mutations occur rarely in patients with lymphoma, although ~1% of patients with classical HL (cHL) carry a reciprocal t(4;9)(q21;p24) translocation that generates a chimeric protein consisting of the proximal end of SEC31A, a protein involved in vesicular transport, fused to the distal half of JAK2 (Figure 1A). The expression of SEC31A/JAK2 enables cytokine-independent proliferation in vitro, and the emergence of an aggressive T-lymphoblastic lymphoma in vivo.

Somatic JAK3 mutations (A572V, A573V) have been identified in 32% of patients with natural killer/T-cell lymphoma (Figure 1B). The A572V substitution provides an in vitro gain-of-function: murine pro-B BaF3 cells no longer require exogenous IL3 for their proliferation, and contain increased levels of phospho-JAK3 and phospho-STAT5. In transduction/transplantation experiments, JAK3A572V expression led to the development of a fatal lymphoproliferative disorder in recipient mice. In addition, FERM domain mutations affecting JAK3 residues L156, R172 or E183 (Figure 1B) were detected in four of 36 patients with adult T-cell leukaemia/lymphoma. When expressed in vitro, these variants enabled cytokine-independent cell growth, with increased levels of phosphorylated JAK3, STAT5 and AKT, demonstrating that they are indeed bone fide gain-of-function mutations. The
mechanism by which these particular variants activate JAK/STAT signalling is not clear, but may be related to the observation that the mutation-bearing JAK3 proteins are significantly more stable in BaF3 cells compared to their wild-type counterpart.\textsuperscript{39}

Despite the paucity of activating JAK mutations in patients with lymphoma, several disease subtypes are characterized by elevated JAK/STAT signalling, suggesting that affected patients have other acquired mutations that perturb this pathway. Indeed, as described in later sections of this review, these affect members of the STAT family, which are the downstream effectors of JAK activation, as well as members of the SOCS and PTPN families, several of which silence activated JAK. Some patients have mutations that perturb the NF-κB pathway, a consequence of which can be the activation of JAK/STAT signalling; in other instances, genomic amplification contributes to lymphomagenesis by causing simple over-expression of JAK2.

\textit{Deregulated JAK/STAT signalling unifies lymphomas with a thymic B-cell origin}

DLBCL is an aggressive malignancy of mature B cells that has a variable clinical course and response to treatment. DLBCL has traditionally proved difficult to sub-classify on the basis of cell morphology, although advances in gene expression profiling over a decade ago have enabled the identification of several distinct groupings,\textsuperscript{40-42} including the “germinal B cell-like” (GCB) and the “activated B cell-like” (ABC) subtypes. Differences in gene expression pattern between these subtypes reflect not only the differentiation stage of the B-cell from which they arose, but also differences in the repertoire of mutations that they bear. Not surprisingly, patients classified according to DLBCL subtype have significant differences in event-free and overall survival rates.\textsuperscript{40, 43}

The molecular signature associated with PMBL is distinct from that of other DLBCL subgroups, but has marked similarities with that of HL.\textsuperscript{41} However, these disease entities are
distinguishable on the basis of their expression pattern of other genes, indicating that the similarities noted are not the consequence of diagnostic inaccuracy, but rather reflect a shared cellular origin: both are considered to have arisen in an ill-defined B-cell population present in the thymic medulla. Although immunophenotypically and histologically distinct, several lines of evidence support the hypothesis that PMBL and HL are, however, pathogenetically related. For example, these disorders have a number of clinical features in common, usually arising in young patients and involving the mediastinum at presentation; an entity termed mediastinal gray zone lymphoma (MGZL) has been identified that has features of PMBL and HL, but cannot readily be assigned to either category.\(^44\) There is also evidence of a disease continuum between the two disorders, with occasional patients initially diagnosed with cHL developing PMBL shortly after treatment. Analysis of matched HL and PMBL samples from three such individuals revealed the presence of common genomic lesions, implying that these disease states were directly related rather than arising separately.\(^45\)

HL and PMBL share a common set of genetic mutations, the most well-characterized of which is a focal amplification of chromosome 9p24, which occurs in approximately 55% of patients with PMBL, 35% of those with HL, and 50% of those with MGZL.\(^45-50\) Comparative genomic hybridization revealed that the common amplified region spanned 3.5Mb and involved 21 genes, ten of which were over-expressed in affected hematopoietic cells.\(^51\) Functional genetic screens then showed that three of these genes, \textit{RANBP6}, \textit{JMJDC2} and \textit{JAK2}, were required for the proliferation or survival of PMBL and HL cell-lines with the 9p24 amplicon.\(^51\) Short hairpin RNA (shRNA)-mediated knockdown of either RANBP6 or JMJDC2 inhibited proliferation but did not cause apoptosis, whereas reductions in JAK2 activity, by either shRNA-mediated knockdown or exposure to a JAK2 inhibitor (TG101348 or AZD1480), induced apoptosis. In both situations, decreased levels of phospho-STAT6 (pSTAT6) were also noted. A similar decrease in pSTAT6 levels occurred when cells were
exposed to IL13 blocking antibodies, which additionally reduced cell surface expression of the IL13 receptor α chain (IL13RA). Collectively, these data suggest that PMBL and HL cell viability is maintained by an autocrine-signalling loop in which JAK2 activity is triggered by IL13 and augmented by the 9p24 amplicon. This causes the phosphorylation and nuclear translocation of STAT6, which binds the regulatory regions of a number of genes, including *IL13*, increasing their transcription. Resulting IL13 protein is secreted, where it binds to an IL13RA subunit on the cell surface, and subsequently enhances JAK2 and STAT6 activation (Figure 2).

What role does RANBP6 or JMJD2 over-expression play in the pathogenesis of PMBL or HL? The biological functions of RANBP6 are not known, although its high (~80%) sequence similarity to importin-5 suggests that it may be involved in nuclear transport. JMJD2 is a Fe- and 2-oxoglutarate-dependent dioxygenase that catalyzes the demethylation of histone H3 lysine-9 (H3K9). Given the role that JAK2 plays in modifying the epigenome, Rui and colleagues evaluated the possibility that these two proteins synergize to relax regions of the genome with a condensed chromatin structure, thereby altering gene expression patterns. shRNA-mediated JMJD2 knockdown sensitized amplicon-positive lymphoma cell-lines to JAK2 pharmacologic inhibition, confirming that JMJD2 and JAK2 co-operate to ensure the survival and expansion of these cells. This dual treatment scheme also increased the number of nuclear foci containing high levels of HP1α, demonstrating that JMJD2 and JAK2 co-operatively suppress heterochromatin formation.

JAK2 and JMJD2 modify histone H3, with JAK2 phosphorylating residue tyrosine-41 and JMJD2 demethylating tri-methylated lysine-9 and lysine-36 (me3K9 and me3K36). HP1α binds to these three residues through its chromo-shadow and chromo domains; their modification inhibits these interactions (Figure 2). Genome-wide analysis of the distribution of phosphorylated H3Y41 in amplicon-positive lymphoma cells showed that the co-operation
between JAK2 and JMJD2 alters the transcription pattern of numerous loci, including the MYC and IL4RA genes, as well as those encoding JAK2 and JMJD2 themselves. Increased synthesis of IL4RA, JAK2 and JMJD2 therefore sets up two positive feedback loops, which enhance autocrine IL13/JAK/STAT-mediated signalling (Figure 2).

**Inactivating SOCS mutations enhance JAK activation in lymphoma cells**

A proportion of PMBL and HL tumours are not positive for the 9p24 amplicon, yet they are characterized by the activation of JAK/STAT signalling, suggesting that, in at least some instances, patients have acquired other mutations that deregulate this pathway. Indeed, some lymphomas carry an acquired mutation in SOCS1, which encodes one of eight members of the suppressors-of-cytokine-signalling (SOCS) protein family. These proteins contain an SH2 domain and a “SOCS box” (Figure 3A) that play pivotal roles in the down-regulation of JAK activity. SOCS proteins bind to the catalytic centre of phosphorylated JAK via their SH2 domain, and recruit Cullin5, Rbx1, elongin-B and elongin-C to their SOCS box. Together, they form an E3 ligase complex that ubiquitinylates the SOCS and JAK proteins, marking them for proteosomal degradation. As SOCS1 gene transcription is enhanced by binding of phosphorylated STAT5 to a site in its upstream regulatory region, SOCS1 forms part of a negative feedback loop that prevents sustained JAK activity induced by the engagement of a cytokine with its cognate receptor. Inactivating mutations in SOCS1 therefore act to enhance or sustain JAK activation.

SOCS1 mutations are found primarily in patients with PMBL or HL. They were first identified in 42% of patients with cHL, and subsequently in a similar proportion of those patients with PMBL or nodular lymphocyte-predominant Hodgkin’s lymphoma (NLPHL). They also occur in a quarter of FL or DLBCL cases, and at lower frequencies in cases of BL, mantle cell lymphoma (MCL) or plasmacytoma. In primary tissue samples, SOCS1
mutations are predominantly mono-allelic, whereas most affected lymphoid cell-lines contain bi-allelic SOCS1 mutations. About half of these mutations result in protein truncation by causing a frame-shift that often adds a variable number of additional (nonsense) amino acids (Figure 3A). In other instances, mutations do not perturb the reading frame, but instead result in an interstitial deletion. These can occur throughout the protein but are concentrated around the SH2 domain and SOCS box, and are predicted to interfere with its ability to bind to phosphorylated JAKs or form a functional E3 ligase complex. In support of either possibility, immunohistochemical analysis of bone marrow samples from those patients with cHL and a SOCS1 mutation showed high levels of phosphorylated STAT5.53 Elevated levels of phospho-STAT6 are similarly associated with the presence of a SOCS1 mutation in patients with NLPHL.55 In addition, JAK2 turnover is significantly impaired in the MedB-1 cell-line, which is derived from a patient with PMBL and carries two mutant SOCS1 alleles.56 Ectopic expression of wild-type SOCS1 in these cells substantially reduced their proliferation rate, with a concomitant decrease in the levels of phosphorylated and total JAK2 and STAT5.

The genetic lesions that increase JAK2 copy number or inactivate SOCS1 do not occur in mutually exclusive subsets of patients. In an analysis of 17 PMBL samples, nine were positive for a SOCS1 mutation, and eight had a wild-type genotype; in both subgroups, the majority was also positive for the 9p24 amplicon (seven and six samples, respectively).55 Rather than providing an alternate mechanism for enhanced JAK/STAT signalling in patients that lack the 9p24 amplicon, SOCS1 mutations may act primarily to boost JAK2 activity even further. A similar situation occurs in acute lymphoblastic leukaemia (ALL), where patients that are positive for an acquired JAK2 mutation often also carry an activating mutation in CRLF2, which is part of the receptor for thymic stromal lymphopoietin, and which signals through JAK2.57,58
Mutations in the PTPN1 or PTPN2 phosphatase sustain cytokine-induced JAK activation

JAK activation can be attenuated by one of two protein tyrosine phosphatases: PTPN1 is responsible for the dephosphorylation of activated JAK2 or TYK2, and PTPN2 for JAK1 or JAK3. Inactivation of PTPN1 or PTPN2 should therefore result in the sustained activity of their associated JAK proteins. Mutations that affect PTPN1 have recently been detected in approximately 20% of primary PMBL and cHL samples; these included missense, nonsense and frame-shift mutations, as well as single-residue deletions (Figure 3B). In numerous cases, co-existent PTPN1 and SOCS1 mutations were detected. Immunohistochemical assessment of tissues from patients with cHL or PMBL revealed that PTPN1 protein levels were reduced in mutation-positive cases. Furthermore, in vitro analyses revealed that all PTPN1 mutants had reduced phosphatase activity compared to wild-type PTPN1, with the impairment level dependent upon the specific mutation present. For example, the Q9 and R156 frame-shift mutants had <10% wild-type PTPN1 activity, whereas the V182D and M282L substitutions had about 30% and 80% activity, respectively. As a consequence of the reduced phosphatase activity, phospho-STAT6 levels induced by exposure of these cells to IL4 remain elevated. Similarly, increased levels of phosphorylated JAK1, JAK2, STAT3, STAT5 and STAT6 were observed in a HL cell-line in which PTPN1 levels were reduced by shRNA-mediated knockdown.

Bi-allelic mutations in PTPN2 can also occasionally occur in patients with HL or T-cell non-Hodgkin’s lymphoma (NHL) (Figure 3C). In almost all cases identified, one entire PTPN2 allele was deleted, whilst the other allele encoded nonsense mutations resulting in severe protein truncation, or amino acid substitutions that are predicted to disrupt an α-helix in the protein tyrosine phosphatase domain. In a single patient, both PTPN2 alleles carried a frame-shift mutation within exon 2; each is predicted to encode a significantly truncated polypeptide (E41fs and R45fs). Introduction of wild-type or mutant PTPN2 into BaF3 cells
revealed that mutant PTPN2 is expressed at substantially lower levels than its wild-type counterpart. However, as might be anticipated, expression of mutant PTPN2 resulted in increased levels of phospho-JAK1, with concomitant increases in levels of phospho-STAT1 and phospho-STAT3, but not phospho-STAT6.

These data collectively suggest that inappropriate activation of JAK/STAT signalling in subsets of patients with lymphoma may result from, or be enhanced by, inactivating PTPN1 or PTPN2 mutations.

**Lymphoma patients may also have acquired mutations affecting a STAT family member.**

Although mutations that target the STAT proteins occur rarely in hematologic malignancies, several studies have recently identified lymphoma patients with an acquired STAT3 mutation, including four of 126 patients with NHL (all of whom had DLBCL) and five of 55 patients with DLBCL. Couronne and colleagues also reported STAT3 mutations in two of 79 DLBCL cases, two of 45 patients with a T-cell lymphoma, and two of ten patients with an anaplastic large cell lymphoma. The affected residues in many instances map to the SH2 domain, although an activating substitution in the coiled-coil domain was reported (Figure 3D). *In vitro* expression of these variants resulted in increased phosphorylation, nuclear translocation, and transcriptional activity of STAT3. An abnormal blood phenotype was apparent in mice transplanted with bone marrow cells expressing the Y640F mutant, although this was myeloproliferative in nature rather than lymphoproliferative.

Ritz and colleagues also identified recurrent heterozygous STAT6 mutations in 36% of patients with PMBL. Each mutation caused an amino acid substitution in the STAT6 DNA binding domain (Figure 3C), primarily affecting residues N417 or D419. These mutations were not restricted to patients lacking the 9p24 amplicon: of the 47 patient samples that were analysed for JAK2, SOCS1 and STAT6 mutations, 20 had mutations affecting one of these
genes, whereas 17 had mutations affecting two, and five had mutations affecting all three. Presence of nuclear phospho-STAT6 in patients with a STAT6 mutation suggested that these may be activating mutations, although nuclear phospho-STAT6 was also observed in most patients that lacked a STAT6 mutation. Surprisingly, in vitro studies suggested that the DNA binding activity of mutated STAT6 was impaired rather than enhanced\textsuperscript{65}; consistent with this finding, crystallographic studies identified the N417 and D419 residues as being located in a DNA recognition element within the STAT polypeptides.\textsuperscript{66,67} Recently, approximately 10% of cases of FL were also found to carry an acquired STAT6 mutation.\textsuperscript{68,69} Almost all of these targeted the DNA binding domain, with residue D419 most commonly affected. Subsequent in vitro studies confirmed that the mutant STAT6 proteins were constitutively activate, with their expression resulting in the transactivation of a STAT6 reporter construct in the absence of exogenous IL4.\textsuperscript{69} Intriguingly, the presence of one of these mutations was not sufficient to cause IL4-independent STAT6 phosphorylation, but instead resulted in an accumulation of non-phosphorylated STAT6 in the nucleus. This in turn resulted in the increased transcription of several loci known to be a direct target of STAT6 (such as CISH, CCL17 and FCER2) in primary lymphoma cells from patients with FL.

**MYD88 mutations affect JAK/STAT signalling in a subset of patients with ABC DLBCL**

Gene expression profiling reveals that the NF-κB signalling pathway is constitutively active in patients with the ABC subtype of DLBCL. This may be as a consequence of a CARD11 mutation (in ~10% of cases)\textsuperscript{70}, an inactivating TNFAIP3 mutation (in almost 25% of cases)\textsuperscript{71}, or constitutively active B-cell receptor signalling arising from CD79A or CD79B mutations (in more than 20% of cases).\textsuperscript{72} However, some patients also have constitutive activation of the JAK/STAT pathway, such that STAT3-mediated signalling synergizes with NF-κB to promote cell survival. The molecular basis of this phenomenon was elucidated in an RNA
interference screen for genes essential for the proliferation or survival of ABC DLBCL cell-lines.\textsuperscript{73} This study identified three independent shRNAs that target MYD88, a signalling adaptor protein that activates the NF-κB pathway in response to stimulation of the toll-like receptors and the IL1 and IL8 receptors (Figure 4). Whole transcriptome sequence analysis of ABC DLBCL cell-lines revealed the presence of a single L265P substitution within MYD88; this variant was also detected in 29\% of ABC DLBCL biopsies, with slightly more than half showing an additional gain of copy number at the mutant \textit{MYD88} locus, suggesting that this mutation may provide lymphoma cells with a dose-dependent competitive advantage. The L265P mutation occurred rarely in the GCB or PMBL subtypes, but was present in 10\% of patients with gastric mucosa-associated lymphoid tissue (MALT) lymphomas and >90\% of patients with Waldenström’s macroglobulinaemia, an IgM-secreting lymphoplasmacytic lymphoma.\textsuperscript{74} Other \textit{MYD88} variants were detected in \~10\% of DLBCL biopsies (both ABC and GCB subtypes); these were mostly located within its TIR domain, which interacts with various growth factor receptors.

\textit{In vitro} experiments revealed that the L265P substitution is a gain-of-function mutation to which mutation-positive ABC DLBCL cell-lines are addicted.\textsuperscript{73} In the absence of signalling through a toll-like receptor or the receptors for IL1 or IL8, mutant MYD88, but not its wild-type counterpart, spontaneously associates with the IRAK1 and IRAK4 kinases, resulting in the phosphorylation of IRAK1 by IRAK4. shRNA-mediated knockdown of IRAK4 levels, or inhibition of its kinase activity by a small molecule inhibitor of IRAK1/4, proved toxic to mutation-positive ABC DLBCL cell-lines. IRAK1 and IRAK4 activation by mutant MYD88 initiates a phosphorylation cascade that results in IκB phosphorylation, and its proteosomal degradation (Figure 4). NF-κB then translocates from the cytoplasm into the nucleus, where it enhances transcription from specific loci. Use of NF-κB reporter constructs in a GCB cell-line engineered to express various \textit{MYD88} species suggests that the L265P,
M232T and S243N mutants were potent inducers of NF-κB signalling, the S22R and T294T variants were moderate inducers, and wild-type MYD88 had minimal activity.

Expression profiling of an ABC DLBCL cell-line both before and after knockdown of mutant MYD88 revealed that, in addition to activation of NF-κB signalling, the JAK/STAT and type I interferon signalling pathways are activated by the L265P substitution mutant. Amongst the differentially expressed transcripts within the NF-κB and JAK/STAT signatures were those for IL6 and IL10. This finding was striking, as autocrine secretion of either cytokine activate JAK1/2- and STAT3-mediated signalling in a significant proportion of patients with the ABC sub-type. shRNA-mediated MYD88 knockdown, or exposure to an IRAK1/4 inhibitor, reduced levels of IL6 and IL10 that were secreted by mutation-positive ABC DLBCL cell-lines, and reduced levels of phospho-STAT3. Acquisition of a MYD88 mutation therefore establishes an autocrine-signalling loop in which nuclear NF-κB enhances the synthesis of IL6 and IL10, which are secreted into the microenvironment. There, these cytokines engage their cognate receptors expressed on the surface of tumour cells, causing activation of JAK1, JAK2 and STAT3. Nuclear phospho-STAT3 then co-operates with NF-κB to enhance IL6 and IL10 synthesis. These cytokines are likely to influence neighbouring non-malignant immune cells, although these effects have not been investigated.

Intriguingly, individuals that carry a (-94ATTG)del polymorphism within their NFKB1 gene promoter have higher plasma levels of IL6 and IL10. Those that develop DLBCL have an inferior progression free and overall survival rate, highlighting the important of the IL6 and IL10 autocrine signalling loops in DLBCL biology.

**JAK/STAT inhibitors as potential new therapeutic agents for the treatment of lymphoma**

As enhanced JAK-mediated signalling is observed in the malignant cells of a significant number of patients with lymphoma, particularly those diagnosed with PMBL or HL, this
signalling pathway should be a focus for the development of novel therapies for patients that may not be adequately served by existing treatment regimens. These new therapies may also benefit other individuals by limiting the adverse side effects associated with conventional radiotherapy and/or chemotherapy. Numerous compounds with JAK inhibitory activity have been created, with several currently being assessed in clinical trials of patients with an MPN, and one (Ruxolitinib; Novartis Pharmaceuticals) having received FDA approval in 2011. Details of those that have been evaluated using lymphoma cells, either in vitro or in vivo, are provided below.

(1) **Fedratinib** (TG101348; Sanofi Pharmaceuticals) has significant selectivity for JAK2 over JAK1 or JAK3 (IC$_{50}$ of <2nM in comparison to 132nM or 250nM, respectively), and also has inhibitory activity against FLT3, TRK and RET. In pre-clinical studies, Fedratinib inhibited the proliferation of primary hematopoietic progenitors from $JAK2V617F$-positive MPN patients.\(^77\) Furthermore, in a murine model of $JAK2V617F$-positive polycythemia vera, Fedratinib treatment reduced the haematocrit, spleen size, and number of erythropoietin-independent erythroid progenitors present in the bone marrow or spleen.\(^78\) In a subsequent Phase I study, Fedratinib reduced splenomegaly in 40% of patients with intermediate-risk or high-risk myelofibrosis, with constitutional symptoms alleviated in the majority of cases.\(^79\) The most frequent hematologic adverse event was myelosuppression.

Unfortunately, Fedratinib’s clinical development has been halted due to unanticipated neurotoxicity. Nevertheless, recently published pre-clinical studies with this drug provide important insights into the clinical utility of JAK inhibitors for the treatment of patients with lymphoma.\(^75,80\) Exposure of 9p24 amplicon-positive cHL and PMBL cell-lines to Fedratinib inhibits their proliferation and viability significantly, with an inverse correlation between 9p24 copy number and the drug’s EC$_{50}$.\(^80\) Fedratinib treatment also reduced levels of MYC
and phosphorylated JAK2, STAT1, STAT3 and STAT6 in a dose-dependent manner. In two different xenograft models, which used the 9p24 amplicon-positive HDLM2 (cHL) or Karpas 1106P (PMBL) cell-lines, exposure to Fedratinib (120mg/kg for 5 days) furthermore caused a significant decrease in phospho-STAT3 levels within tumour cells, decreased their rate of growth, and prolonged survival of these animals.

(2) AZD1480 (AstraZenca Inc.) is a pyrazol pyrimidine ATP-competitive inhibitor of JAK1 and JAK2 with IC_{50} values of 1.4nM and 0.4nM, respectively. At higher concentrations, it also inhibits the activities of JAK3 and TYK2, and Aurora-A kinase. Treatment of HL cell-lines with varying doses of AZD1480 has shown that, at low doses (0.1-1.0nM), levels of phospho-STAT1, phospho-STAT3, phospho-STAT5 and phospho-STAT6 were all reduced significantly, although rates of proliferation was unaffected. At doses above 1nM, however, AZD1480 induced G2/M cell cycle arrest and apoptosis, as the result of inhibition of Aurora A kinase activity. Clinical evaluation of AZD1480 in patients with lymphoma has not yet been reported.

(3) Pacritinib (SB1518; Cell Therapeutics). The first of the Phase I trials to evaluate the safety and efficacy of various JAK inhibitors in patients with lymphoma involved the use of Pacritinib, a macrocyclic pyrimidine-based inhibitor with activity against both wild-type and mutant JAK2 (with an IC_{50} of ~20nM), and FLT3. Thirty-four patients with refractory or relapsed lymphoma (HL, NHL, FL and MCL) received at least one dose of Pacritinib; seventeen were treated for at least three months, and six for at least six months. Thirty-one of these patients had pre- and post-baseline computed tomography (CT) scans to evaluate disease status; seventeen (55%) showed decreased tumour measurements following Pacritinib treatment, ranging from 4% to 70%. Importantly, molecular assessment of mutations in JAK2, or others that deregulate JAK/STAT signalling, was not performed. It is therefore unclear what effect, if any, Pacritinib has on the size of the malignant clone.
(4) **Ruxolitinib** (Jakafi™) is a pyrrolo [2,3-d]pyrimidine analogue that exhibits nanomolar affinity for both JAK1 and JAK2 (with IC$_{50}$ values of 2.7nM and 4.5nM, respectively). In a multi-centre study of 153 patients with myelofibrosis, this drug was well tolerated and had beneficial effects against splenomegaly and constitutional symptoms. Two randomized Phase III trials in myelofibrosis patients were subsequently reported: COMFORT-I, a placebo-controlled trial of 309 patients; and COMFORT-II, a comparison of Ruxolitinib to best available therapy in 219 patients. Ruxolitinib significantly affected splenomegaly, with almost all patients experiencing a reduction in volume, and overall improvement in quality-of-life was reported in half of those receiving Ruxolitinib. The most frequent adverse events were anaemia or thrombocytopenia, which could be managed by a dose reduction or brief interruption in treatment. A Ruxolitinib-mediated reduction in circulating pro-inflammatory cytokine levels is attributed to its inhibitory effects on JAK1-mediated signalling; this may make Ruxolitinib an attractive therapeutic agent for MYD88 mutation-positive lymphoma patients, as this mutation causes cell-autonomous activation of JAK1 and JAK2 (Figure 4). However, the effects of Ruxolitinib on primary cHL and/or PMBL cell proliferation *in vitro* or *in vivo* have not yet been reported.

These JAK inhibitors might be used in conjunction with existing therapies, or with other agents that also inhibit JAK2 activity, such as the HSP-90 inhibitors. Alternately, drugs that target particular downstream effectors of activated JAKs, such as those that inhibit STAT activity, should be considered. Although there is little published data on the efficacy of STAT inhibitors on lymphoma cells, one such agent, OPB-31121 (Otsuka Pharmaceuticals), had a strong inhibitory effect on the growth of all BL cell-lines tested (n=3) and in five of nine DLBCL cell-lines, in addition to a number of other hematologic malignancies. Phase I/II studies in patients with NHL (NCT1406574) are underway.
Is JAK/STAT inhibition beneficial for all lymphoma sub-types?

Given that a sizeable proportion of lymphoma patients show activation of the JAK/STAT signalling, especially those with the PMBCL and HL sub-types, it is reasonable to question whether all lymphoma sub-types might be amendable to treatment with a JAK inhibitor. However, a recent study has suggested that JAK2 activation plays a key role in maintaining control of B-cell proliferation in the germinal centre (GC).\textsuperscript{20} As a consequence, those lymphoma sub-types that arise following the transformation of a germinal B-cell, such as BL, Fl and GC-DLBCL, are not predicted to benefit from the inhibition of JAK2 activity.

In naïve B-cells and most somatic cells, expression of the lysine methyltransferase, EZH2, is absent. However, levels are significantly increased in primary lymphoid follicles upon B-cell activation and formation of the GC. Chromatin immunoprecipitation experiments suggest that EZH2 binds to 1800 gene loci within GC cells,\textsuperscript{88} catalysing the formation of the transcriptionally repressive H3K27me2 and H3K27me3 epigenetic marks (Figure 5). As a consequence, cell cycle checkpoints are silenced and rapid B-cell proliferation occurs. In later stages of development, proliferation rates decrease as a result of a reduction in EZH2 levels, a process initiated by the activation and nuclear translocation of JAK2, which enables EZH2 phosphorylation on tyrosine-641 (Y641).\textsuperscript{20} Phosphorylated EZH2 is a substrate for the β-TrCP-containing SCF E3 ubiquitin ligase complex, initiating the polyubiquitinylation of EZH2 and its eventual proteosomal degradation (Figure 5).\textsuperscript{20}

Any factor that prevents the JAK2-mediated degradation of EZH2 may therefore enable the sustained proliferation of germinal B-cells. An example of this phenomenon is provided in nature: gain-of-function mutations in EZH2 have been detected in a significant proportion of patients with GC-DLBCL or FL, but are extremely rare in other lymphoma subtypes or acute leukaemias\textsuperscript{89, 90} and impair its degradation. Most of these affect Y641, although substitutions of A677 or A687 can occur; all three residues map to the SET domain, which
mediates its methyltransferase activity. Biochemical analyses have revealed that mutant EZH2 expression is associated with elevated levels of H3K27me3.\textsuperscript{89–91} Two selective inhibitors of EZH2 have been tested on lymphoma cells\textsuperscript{92, 93}; each significantly impaired H3K27 methylation and cell proliferation \textit{in vitro}, and one significantly extended the lifespan of mice xenografted with human EZH2-mutant lymphoma cells, demonstrating that drugs that inhibit EZH2 activity may have clinical utility.

Treatment of EZH2-wildtype GC-DLBCL, BL or FL cells with Ruxolitinib would similarly serve to prevent nuclear accumulation of JAK2 and thereby increase EZH2 levels, mimicking the effects of an EZH2 mutation. Fortunately, data from transgenic mice that express Ezh2Y641F suggest that sustained levels of EZH2 promote a hyperproliferative state, but not overt B-cell transformation.\textsuperscript{94} Treatment of patients with these lymphoma subtypes would therefore prove to not be beneficial, but may also not cause harm.

\textit{Concluding comments}

Gene expression profiling and genomic sequence determinations performed over the last decade have clearly implicated activation of the JAK/STAT pathway in the pathogenesis of several lymphoma sub-types, including diffuse large B-cell lymphoma, Hodgkin lymphoma, and primary mediastinal B-cell lymphoma. Although \textit{JAK2} coding sequence mutations are extremely rare in patients with these malignancies, a variety of mutational events have now been identified that cause over-expression of non-mutated \textit{JAK2}, target other components of the JAK/STAT pathway, or establish an autocrine signalling loop that drives JAK-mediated cytokine-independent proliferation. The observation that some patients may carry three or more distinct mutations that each activate JAK/STAT signalling highlights the importance of this pathway in lymphomagenesis. Collectively, these studies provide a rationale for clinical trials of JAK inhibitors in patients with lymphoma, although studies should be confined to
those individuals with an activated JAK/STAT “signature”. A handful of currently available JAK inhibitors, including Ruxolitinib and Pacritinib, may prove useful in the treatment of lymphoma patients that are not adequately served by existing therapies. The development of second-generation JAK inhibitors, which are anticipated in light of recent advances in our understanding of the structure and auto-regulation of the JAK polypeptides,\textsuperscript{10-12} should prove to be even more beneficial.
Conflict of Interest Statement

None.

Acknowledgments

This work was supported by funds from the University of Queensland Diamantina Institute. LMS would like to dedicate this review to the memory of Beryl Lloyd, friend and teacher.
References


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Figure 1. JAK2 mutations are rare in lymphoma cases, although JAK3 mutations can occur in patients with natural killer/T-cell lymphoma. (A) JAK2 mutations are frequently found in patients with a myeloproliferative neoplasm (MPN), either as a V617F substitution or as deletions, substitutions or insertions of residues that are encoded for by JAK2 exon 12. A third JAK2 mutation category (R683) is frequent in cases of paediatric acute lymphoblastic leukaemia. In contrast, mutations within JAK2 are not frequently present in patients with lymphoma, although a small percentage of those with classical Hodgkin’s lymphoma bear a t(4;9) translocation that generates a SEC31A-JAK2 chimaeric protein. The SEC31A portion of the chimaera is depicted here in dark grey. (B) Despite significant sequence homology between the JAK2 and JAK3 polypeptides, lymphoma-associated JAK3 mutations do not affect residues analogous in position to those mutated in JAK2. Instead, residues A572 and A573 in the JH2 domain, and L156, R172 and E183 in the FERM domain, are mutated.

Figure 2. Feed-forward signalling loops in lymphoma cells bearing the 9p24 amplicon.
In lymphoma cells containing the 9p24 amplicon, the over-expression of wild-type JAK2 protein causes activation of canonical JAK/STAT signalling. As a consequence, phosphorylated STAT6 translocates from the cytoplasm into the nucleus, where it binds to STAT consensus binding sites within the regulatory regions of target genes, enhancing their transcription. Amongst these is the one encoding interleukin-13 (IL13). As these cells express functional IL13 receptors, which are heterodimers consisting of an IL4RA chain and an IL13RA chain, the production of IL13 causes autocrine stimulation of JAK/STAT signalling. Non-canonical JAK-mediated signalling is also at play in amplicon-positive lymphoma cells. Activated JAK2 can translocate into the nucleus, where it phosphorylates tyrosine-41 of the

Figure Legends

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histone H3 and prevents the binding of heterochromatin protein-1α (HP-1α) at this site. Jumonji (JMJD2C) similarly alters epigenetic marks, removing trimethyl moieties from histone H3 lysines-9 and -36. Therefore, JAK2 and JMJD2C synergize to relax heterochromatic regions of the genome, enhancing transcription of functionally important genes. These include the JMJD2C and JAK2 loci, setting up a second positive feedback loop. IL4RA transcription and synthesis is also increased, furthering enhancing autocrine IL13/JAK/STAT-mediated signalling.

**Figure 3. Mutations in SOCS, PTPN or STAT proteins enhance JAK activation.** (A) The SOCS family of adaptor proteins includes four functionally important domains: the kinase inhibitory region (highlighted by hatching), extended SH2 domain (ESS; in black), SH2 domain (light grey), and the “SOCS box” (dark grey). SOCS proteins bind to the catalytic centre of a phosphorylated JAK protein via their SH2 domain, and the SOCS box provides a docking site for elongin-B, elongin-C, Cullin5 and Rbx1, causing the proteosomal degradation of JAK and SOCS proteins by mediating their ubiquitinylation. A sampling of SOCS1 mutations reported in the literature is shown. Half are protein truncations caused by the introduction of a STOP codon (for example, L115X) or a loss of nucleotides that shifts the reading frame. In the latter instance, this may also add a variable number of “nonsense” residues, indicated by dashed lines. In other instances, loss of nucleotides instead produces a focal deletion that invariably involves part of the SH2 domain and/or the SOCS box. In rare examples, amino acids not present in the wild-type SOCS1 protein are inserted (an example of which is indicated by the cross-hatched region of SOCS1(79-91)ins,(157-160)del. (B) PTPNI is responsible for the dephosphorylation of activated JAK2 or TYK2. Approximately 20% of patients with primary mediastinal B-cell lymphoma or Hodgkin’s lymphoma have missense, nonsense and frame-shift PTPN1 mutations, or single-residue deletions. These
impair phosphatase activity, such that increased levels of phosphorylated JAK1, JAK2, STAT3, STAT5 and STAT6 are present in mutation-positive cells. (C) Hodgkin’s lymphoma or T-cell non-Hodgkin’s lymphoma can be associated with biallelic PTPN2 mutations. These may impair phosphatase activity, as well as significantly reduce protein expression levels. As a consequence, affected cells have increased levels of phospho-JAK1, with phospho-STAT1 and phospho-STAT3. (D) A small proportion of lymphoma patients may carry an acquired STAT3 mutation. The affected residues mostly map to within the SH2 domain (in light grey), although an activating M206K substitution has been reported in the coiled-coil domain. In contrast, the acquired mutations affecting STAT6, which occur in ~35% of patients with primary mediastinal B-cell lymphoma, occur within the DNA binding domain (DBD; highlighted in dark grey), but not the SH2 domain. Arrowheads indicate the location of various lymphoma-associated STAT3 and STAT6 mutations.

**Figure 4. MYD88 mutation induces autocrine signalling mediated by IL6 and IL10.**

Mutations in MYD88 (shown here in green) cause the spontaneous formation of a complex between it and the IRAK1 and IRAK4 serine/threonine kinases. In healthy cells, this complex is assembled only after MYD88 interacts with a Toll-like receptor (TIR) or the IL1 receptor. IRAK4 is then within close proximity to IRAK1, and is able to phosphorylate it, initiating a signalling cascade that includes the phosphorylation of TAK1, the α and β chains of I-kappa-B kinase (IKK), and IκB. This ensures the ubiquitinylation and proteosomal degradation of IκB, which serves to inhibit and maintain within the cytoplasm the NF-κB transcription factor. MYD88 mutation ultimately allows the nuclear translocation of NF-κB, which then binds to specific regulatory elements within target genes, and enhances their transcription. Amongst the loci directly affected are those that encode IL6 and IL10, setting an autocrine-signalling loop via their cognate receptors on the cell surface. Signalling through either
receptor results in JAK-mediated phosphorylation and nuclear translocation of STAT3, which also enhances transcription of the *IL6* and *IL10* genes.

**Figure 5. JAK2 inhibition and EZH2 mutation have the same effects in germinal B-cells.**

In naïve B cells, and most somatic cells, expression of the lysine methyltransferase, EZH2, is absent. However, EZH2 expression is significantly up-regulated in primary lymphoid follicles during B-cell activation and germinal centre (GC) formation. There, EZH2 catalyses the di- and tri-methylation of histone H3, forming the transcriptionally repressive H3K27me2 and H3K27me3 epigenetic marks (collectively indicated here by a green circle labelled “M”). As a consequence, cell cycle checkpoints are silenced and rapid B-cell proliferation occurs. In later stages of B-cell development, EZH2 is once more silenced, permitting exit from the GC and subsequent differentiation. This process is initiated by the activation and nuclear translocation of JAK2, and by its phosphorylation of EZH2 on tyrosine-641 (Y641). Phosphorylated EZH2 is a substrate for the β-TrCP SCF E3 ubiquitin ligase complex, which initiates the polyubiquitination of EZH2 (indicated here as a brown circle labelled “U”), and its eventual proteosomal degradation. JAK2-mediated degradation of EZH2 is impaired in a significant proportion of GC-DLBCL cases by substitution mutations affecting Y641. As a consequence, EZH2 levels remain high, promoting a hyperproliferative state but not overt B-cell transformation. *In vitro* treatment of non-lymphoid somatic cells with Ruxolitinib similarly increases EZH2 levels, suggesting that there is likely to be no benefit to treating DLBCL patients with JAK inhibitors.
Figure 1: Proteins involved in the JAK-STAT pathway.

A. Protein domains:
- JAK2: FERM, SH2, JH2, JH1
- V617F
- Exon 12
- R683
- SEC1A/JAK2

B. Domain comparison:
- JAK2: FERM, SH2, JH2, JH1
  - % identity: 66%, 65%, 76%, 77%
- JAK3
- A572V
- A573V
- FERM

FIGURE 1, SCOTT & GANDHI
FIGURE 2, SCOTT & GANDHI
Figure 3
FIGURE 4, SCOTT & GANDHI
Nuclear translocation of phospho-JAK2 results in EZH2 phosphorylation and recruitment of E3 ubiquitin ligase complex

EZH2 ubiquitylation and proteasomal degradation results in relaxation of chromatin structure and target gene expression

FIGURE 5, SCOTT & GANDHI