In Vivo Activation of Signal Transducer and Activator of Transcription 1 after CD154 Gene Therapy for Chronic Lymphocytic Leukemia Is Associated with Clinical and Immunologic Response

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ABSTRACT

Purpose: Signal transducer and activator of transcription (STAT) proteins are important regulators of physiological stimuli in lymphocytes. Biological therapies directed at lymphocytic malignancies such as chronic lymphocytic leukemia (CLL) may be mediated by these transcription factors. One such approach, CD154 (CD40-ligand) gene therapy, involves expressing CD154 on malignant B cells from CLL patients by transduction with an adenovirus vector after which the cells are reinfused into the patients. To determine the intracellular signaling pathways that underlie the clinical and immunological responses observed in patients from a Phase I study of CD154 gene therapy, CLL cells from these patients were examined for changes in STAT signaling events.

Experimental Design: CLL cells from patients who underwent CD154 gene therapy were analyzed for changes in STAT signaling by Western blot analysis and electrophoretic mobility shift assay. Activation of STAT1 was correlated with patient response to therapy.

Results: Tyrosine phosphorylation of STAT1 was detected in the nontransduced CLL cells in 9 of 11 patients 24 h after infusion, but not before. Activation of STAT1 was associated with clinical response, as measured by decreased absolute lymphocyte count, and immunological response, as measured by elevated plasma levels of IFN-γ.

Conclusion: This study indicates that STAT signaling may be an important mediator of biological treatments, such as CD154 gene therapy, and that early STAT1 activation may predict response to this novel treatment.

INTRODUCTION

CLL, the most prevalent leukemia in Western countries, is a malignancy of mature B lymphocytes, which accumulate in the Go phase of the cell cycle. Currently there is no curative therapy for CLL, although a number of promising new therapies are under investigation. Among these are immune-based strategies designed to generate an immune response against the malignant B lymphocytes. CLL cells are poorly immunogenic, possibly caused by ineffective antigen presentation or because of defects in T-cell recognition and effector function (1–4). A Phase I study was conducted in which CLL cells were transduced ex vivo with adenovirus to express murine CD154 (Ad-CD154-CLL cells; Ref. 5). The transduced CLL cells were reinfused into patients to facilitate induction of an immune response to CLL. Eleven patients with CLL were included in the trial. Clinical responses to the CD154-CLL gene therapy included decreases in circulating leukemic cells (reflected in decreased peripheral blood lymphocytes and total WBCs) as well as decreased lymphadenopathy. Patients also experienced immunological responses characterized by elevated cytokine levels (IFN-γ, IL-12, and IL-6) and increased absolute T-cell counts. A critical question arising from this study is the nature of the intracellular events that mediate the response to this therapy.

Although the etiology of CLL remains unknown, a number of molecular aberrations have been identified (6–10). Among these, STAT proteins are known to be constitutively phosphorylated on serine residues in CLL cells but not in normal B lymphocytes (11). In response to cytokines, growth factors, and hormones, STAT proteins mediate a number of important biological responses in lymphocytes, including cell growth, differentiation, and survival. Janus-activated kinases and other tyrosine kinases phosphorylate a single tyrosine residue to activate STAT proteins. Tyrosine phosphorylation leads to homo- or heterodimerization of STAT dimers, which then translocate to the nucleus to regulate gene transcription.

The abbreviations used are: CLL, chronic lymphocytic leukemia; IL, interleukin; STAT, signal transducer and activators of transcription; PMSF, phenylmethanesulfonyl fluoride; EMSA, electrophoretic mobility shift assay; ALC, absolute lymphocyte count.

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heterodimerization of STAT monomers and resultant nuclear translocation. STAT dimers subsequently bind specific DNA sequences in the promoters of target genes and modulate transcription. Some STATs can also become phosphorylated on serine residues. The nature of the constitutive serine phosphorylation in CLL is not well understood, but it is thought to enhance transcriptional activation and/or promote interactions with other intracellular proteins.

Because STAT signal transduction mediates many of the physiological responses of stimuli in lymphocytes, CLL cells from the patients in the CD154 gene therapy trial were examined for STAT signaling events that could mediate the clinical responses observed in these patients. The level of tyrosine-phosphorylated STAT1 increased in the majority of patients given CD154 gene therapy, attributable, at least in some patients, to the production of a soluble factor. Moreover, there was an association between STAT1 activation and clinical response, indicating that STAT1 may be an important mediator of CD154 gene therapy and a predictor of response.

RESULTS
Quantification of STAT Activation. Because STAT1 activation can lead to up-regulation of STAT1 protein levels, phosphorylated STAT1 was normalized to tubulin of which the expression is invariant in CLL cells. After immunoblotting with antityrosine-phosphorylated STAT1 antibodies, blots were stripped and reprobed using a 1:5000 dilution of antitubulin antibody (clone B-5–1-2; Sigma, St. Louis, MO) in Tris-buffered saline/0.05% Tween 20 and processed as described above. Films from the immunoblots were scanned, and mean intensities were calculated using Kodak Digital Science 1D image analysis software. The ratio of tyrosine phosphorylated STAT1s, the transcriptionally active form of STAT1, to tubulin was then calculated for patients before treatment and after 24 h of treatment.

EMSA. Nuclear extracts were prepared by washing cells once with cold PBS, resuspending in hypotonic buffer [10 mM Tris (pH 7.4), 10 mM NaCl, 6 mM MgCl2, 1 mM β-mercaptoethanol, 1 mM sodium orthovanadate, 10 μg/ml PMSF, 2 μg/ml pepstatin, 2 μg/ml leupeptin, and 10 μg aprotinin], and incubating on ice for 5 min. The cells were centrifuged for 10 s at 12,000 × g and then resuspended in hypotonic buffer. Cells were disrupted using 30 strokes with a Dounce homogenizer (Type B pestle). The nuclei were collected by centrifugation for 10 s at 12,000 × g and washed once with hypotonic buffer. The nuclear pellet was then resuspended in 1 pellet volume of high salt buffer [20 mM HEPES (pH 7.9), 420 mM NaCl, 1.5 mM MgCl2, 25% glycerol, 0.2 mM EDTA, 10 μg/ml PMSF, 1 mM sodium orthovanadate, and 1 mM β-mercaptoethanol] and incubated for 30 min at 4°C with shaking. The nuclear extract was clarified by centrifugation for 3 min at 12,000 × g. A double-stranded 32P-labeled oligonucleotide (1 ng) derived from the ciliary neurotrophic factor response element (5′- CGCCCTGATTCTCCCCGGAATGACGGCG-3′ and its complement; Ref. 13) was incubated with 2 μl of nuclear extract in 10 μl binding buffer [25 mM HEPES (pH 7.9), 100 μg/ml EDTA, 200 μM MgCl2, 500 μM DTT, 1 μg/ml BSA, 0.2 μg/μl poly(deoxyxynosinic-deoxycytidylic acid), 1% Ficoll, and 0.1 μg/μl salmon sperm DNA] for 15 min at room temperature. Supershift analysis was performed by incubating the nuclear extracts with 1 μg anti-STAT1 antibody (SC-346; Santa Cruz Biotechnology) for 20 min on ice before adding the binding buffer. The protein-DNA complexes were separated by electrophoresis on a 4% polyacrylamide gel in 0.2× Tris-borate/EDTA. The gel was dried and exposed to film.

Statistical Analysis. The Wilcoxon rank sum test was used for statistical significance between two groups, and paired data were evaluated by the Wilcoxon signed rank test. Ps were two-sided.

RESULTS
CD154 Gene Therapy Induces Tyrosine Phosphorylation of STAT1 in Vivo. We examined the CLL cells from the 11 patients in the Phase I study of this therapy and analyzed the intracellular signaling pathways that were modulated during treatment. Whole cell lysates from CLL cells collected before and 24 h after infusion with Ad-CD154-CLL cells were analyzed by SDS-PAGE followed by immunoblotting using antibodies that specifically recognize the tyrosine-phosphorylated
form of STAT1. Western analysis revealed that in 9 of 11 patients, CD154 gene therapy caused tyrosine phosphorylation of STAT1. CLL cells isolated 24 h after CD154 gene therapy had increased levels of tyrosine phosphorylated STAT1 compared with cells obtained before therapy (Fig. 1A, top). The cells of 1 patient were only available after treatment at which time there was weak but noticeable STAT1 activation (data not shown). Both the α (Mr 91,000) and β (Mr 84,000) isoforms of STAT1 are expressed in these cells. Although both forms can be tyrosine phosphorylated, only the α form can be serine phosphorylated, as the β form lacks the COOH-terminal serine phosphorylation site. Blots were stripped and reprobed with an antibody that recognizes the serine-phosphorylated form of STAT1. CD154 gene therapy had no effect on the constitutive serine phosphorylation of STAT1 (Fig. 1A, middle).

Given the importance of STAT3 and STAT5 in transducing signals in lymphocytes, cells were also analyzed for activation of these transcription factors. No induction of tyrosine phosphorylation of STAT3 or STAT5, or change in the constitutive serine phosphorylation of STAT3 was observed (data not shown).

After reinfusion of the CD154-transduced CLL cells, the transduced cells make up 0.01% of the total CLL population. Nonetheless, it is possible that adenovirus infection of these cells directly leads to tyrosine phosphorylation of STAT1. To verify that infection of CLL cells with Ad-CD154 did not cause STAT1 activation, Western analysis was performed on lysates from cells that were infected with Ad-CD154, a control adenovirus encoding an irrelevant protein (β-galactosidase; Ad-lacZ) or left uninfected. No STAT1 activation was detected in Ad-CD154-infected, Ad-lacZ-infected, or uninfected CLL cells (data not shown).

Consistent with this observation is the finding that STAT1 phosphorylation is not detected in CLL cells 0.5 h after infusion of Ad-CD154-CLL cells (Fig. 2B, Lane 5).

To confirm that tyrosine-phosphorylated STAT1 had localized to the nucleus and could bind to consensus STAT1 binding sites, EMSA was used. Because of the limited number of cells available, it was possible to perform EMSA on cells from only 2 patients. Nuclear extracts were incubated with a radiolabeled STAT1-response element and EMSA was performed. The results showed that STAT1 binds to the STAT1-response element, as expected.

Fig. 1 CD154 gene therapy induces tyrosine phosphorylation of STAT1 in vivo. A, Western blot analysis was performed on whole cell lysates from CLL cells obtained from patients before (Pre) and 24 h after infusion of Ad-CD154-CLL cells. Blots were probed with antibodies to tyrosine-phosphorylated STAT1 (top), or serine-phosphorylated STAT1 (middle), and total STAT1 (bottom). Data from the 4 patients shown are representative of the 9 patients whose cells demonstrated STAT1 activation. B, DNA binding activity of nuclear extracts from CLL cells before (Pre) and 24 h after patients were infused with Ad-CD154-CLL cells was examined by EMSA. Antibody against total STAT1 (αS1) was added to extracts before incubation with radiolabeled probe. Data from the 1 patient shown are representative of the 2 patients for whom sufficient cells were available for analysis. C, Western blots probed with antityrosine-phosphorylated STAT1 antibody (Fig. 1A) were stripped and reprobed with antitubulin antibody. The levels of tyrosine phosphorylated STAT1α (the transcriptionally active form of STAT1) were quantified and normalized to tubulin, and the ratio of tyrosine phosphorylated STAT1α to tubulin was calculated for patients before treatment and 24 h after treatment (P = 0.002).

Fig. 2 Kinetics of STAT1 tyrosine phosphorylation. A, whole cell lysates were prepared from CLL cells collected from patients before treatment (Pre) with Ad-CD154-CLL cells or 1–7 days after treatment and analyzed by Western blotting with an antibody to tyrosine-phosphorylated STAT1. Similar results were obtained in 2 additional patients. B, whole cell lysates were prepared from CLL cells collected from patients before treatment (Pre), 0.5 h, or 48 h after treatment and analyzed by Western blotting with an antibody to tyrosine-phosphorylated STAT1. Blots were stripped and reprobed for total STAT1.
probe containing a STAT1 binding site and analyzed by gel electrophoresis. A STAT1-DNA complex formed from nuclear extracts of cells obtained 24 h after therapy but not from cells obtained before treatment (Fig. 1B, complex III). This complex was disrupted by prior incubation of the nuclear extract with an antibody that recognizes STAT1, confirming the presence of this transcription factor. Two additional non-STAT1-DNA complexes were observed (Fig. 1B, complexes I and II), but these were present in cells before treatment and were unaffected by the gene therapy or by addition of the STAT1 antibody.

To convert the biochemical data into a numerical metric, the levels of tyrosine-phosphorylated STAT1 before treatment and 24 h after treatment were normalized to tubulin by image analysis. The cells of 1 patient were only available 8 h after treatment. Therefore, only cells from 10 of the 11 patients were included in the comparison. In the CLL patients treated with CD154 gene therapy, there was a significant increase in the ratio of tyrosine phosphorylated STAT1 to tubulin after 24 h ($P = 0.002$; Fig. 1C).

**Kinetics of in Vivo STAT1 Activation.** The immunological and clinical responses to Ad-CD154 gene therapy take place over a period of days (5). To assess the activation of STAT1 during this time frame, CLL cells were collected from patients before and up to 7 days after infusion with Ad-CD154-CLL cells. Western analysis revealed that Ad-CD154-CLL infusion induced tyrosine phosphorylation of STAT1 within 24 h, and this phosphorylation was maintained for several days (Fig. 2A). By 7 days after infusion, STAT1 phosphorylation had returned to basal levels. Thus, in vivo activation of STAT1 after this therapy shows prolonged kinetics compared with the transient activation usually seen with in vitro systems (14).

Seven of the 11 patients in the Phase I trial subsequently received repeated infusions of Ad-CD154-CLL cells. To test whether STAT1 activation occurs with repeated doses of Ad-CD154-CLL cells, CLL cells were examined for STAT1 tyrosine phosphorylation before and up to 48 h after each infusion in 1 patient who received three infusions of Ad-CD154-CLL cells. Six months elapsed between the first and second infusions, and 3 weeks elapsed between the second and third infusions. Although no tyrosine phosphorylation of STAT1 was detected before or immediately after each infusion, STAT1 was tyrosine phosphorylated after each of the three Ad-CD154-CLL infusions (Fig. 2B). These data indicate that STAT1 becomes activated after infusion, subsequently dephosphorylated, and can be reactivated with each successive infusion. Thus, STAT1 activation occurs in a reproducible manner in response to repeated doses of CD154 gene therapy.

**Association between STAT1 Activation and Therapeutic Responses.** To determine whether the STAT1 activation in patients given CD154 gene therapy was clinically relevant, the level of STAT1 activation was compared with two biological endpoints. In the Phase I trial, therapeutic outcome was clinically defined in part by a decrease in the ALC. Therapeutic response was also defined immunologically by increased Th1 cytokine levels, which reflected a host response to the CD154 gene therapy. To determine whether there was an association between STAT1 activation and either clinical or immunological response, the percentage of decrease in ALC or the increased level of the Th1 cytokine IFN-γ was compared between patients with and without STAT1 activation (Table 1). STAT1 activation was defined as ≥3-fold increase in STAT1 tyrosine phosphorylation, a level thought to be sufficient for transcriptional induction, after Ad-CD154-CLL cell infusion. The percentage of decrease in ALC was determined by comparing the ALC immediately before Ad-CD154-CLL cell infusion and the ALC nadir 2–4 days after treatment. Whereas there was not a linear correlation between the magnitude of STAT1 activation and clinical response, the 9 patients who experienced ≥20% decrease in ALC also demonstrated STAT1 activation ($P = 0.04$; Fig. 3A). Nearly identical decreases in the percentage of WBCs were observed in these patients as well. Thus, patients who experienced decreased WBC counts also demonstrated STAT1 activation (data not shown). Similarly, higher levels of IFN-γ (median, 0 ng/ml versus 490 ng/ml; $P = 0.07$) were found in patients who had increased levels of STAT1 activation (Fig. 3B). Moreover, the patient whose cells were analyzed for STAT1 activation after each repeated dose of Ad-CD154-CLL cells (Fig. 2B) experienced a decrease in ALC with each infusion (30%, 41%, and 59% after infusions 1, 2, and 3, respectively, relative to that before each infusion), and an increase in IFN-γ 24 h after the second and third treatments (0 ng/ml, 1200 ng/ml, and 200 ng/ml after infusions 1, 2, and 3, respectively). These data suggest that STAT1 activation may be an early marker of response to this therapy and may potentially mediate the clinical response.

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**Activation of STAT1 by Plasma from Patients Treated with CD154 Gene Therapy.** Given the association between STAT1 activation and therapeutic response, the mechanism by which STAT1 became activated after CD154 gene therapy was investigated. Because STAT1 phosphorylation was associated with Th1 cytokine production, we considered the possibility that this activation might be initiated by extracellular ligands (cytokines, hormones, or growth factors) secreted into the plasma in response to therapy. Untreated autologous CLL cells were incubated with plasma for 15 min, a time at which cytokines induce maximal STAT phosphorylation in vitro. Plasma obtained 24 h after treatment with Ad-CD154-CLL cells strongly
activated STAT1 in the cells of 2 of 7 patients, indicating that,

at least in these patients, STAT1 activation was induced by a
factor acting in an autocrine or paracrine manner (Fig. 4). In
these in vitro experiments, plasma from patients 3 and 8 caused
STAT1 activation, whereas plasma from patients 4, 7, 9, 10, and
11 did not, although each of these patients experienced activa-
tion of STAT1 in vivo (Table 1). These findings also indicate
that the activating factor is not present before or 4 days after
infusion. Findings in the cells of the remaining 5 patients ex-
pressed were inconclusive, and the limited plasma available
precluded additional characterization.

It was suggested recently that IL-15 contributes to the
immunological effects of CD154 gene therapy (15). Considering
that the IL-15 receptor includes the γc chain, which is shared by
a number of other cytokine receptors that signal through
STAT1, we tested the possibility that IL-15 could be responsible
for STAT1 activation. CLL cells from 3 patients not on the
CD154 gene therapy trial were incubated with 10 ng/ml IL-15
for 15 min in vitro. Whereas there was weak activation of
STAT1, there was also activation of STAT5, which was never
observed in the patient cells after 24 h of CD154 gene therapy
(data not shown). Although this does not rule out the possibility
that IL-15 mediates later signaling events and therapeutic ef-
teffects, it is not the factor responsible for the rapid activation of
STAT1 observed within 24 h of therapy.

Because plasma-induced activation of STAT1 was not ob-
served in all of the patients, we tested the possibility that STAT1
activation could be caused by direct contact between the Ad-
CD154-transduced CLL cells and untransfected “bystander”
CLL cells. It is known that cell-cell contact can activate STAT1
in lymphocytes through other cell surface molecules (14). In the
treated patients, the number of bystander CLL cells greatly
exceeded the infused Ad-CD154-CLL cells by a ratio of
>10,000:1 (5). To determine whether cell-cell interaction could
induce STAT1 activation, uninfected CLL cells and CLL cells
infected with Ad-CD154 for 24 h were incubated together at
ratios ranging from 10,000:1 to 1:1 and then analyzed for
STAT1 activation by Western analysis. No STAT1 activation
was detected in any mixture of cells (data not shown) indicating
that direct cell-cell contact between the Ad-CD154-transduced
cells and bystander CLL cells cannot account for the STAT1
activation.

**DISCUSSION**

In the present study, we demonstrate that CD154-CLL gene
therapy induces tyrosine phosphorylation and functional activa-
tion of STAT1 in vivo. The activation of STAT1 was associated
with clinical and immunological benefits as measured by de-
creased ALC and increased cytokine production. These data
suggest that STAT1 activation may be important in the therapeutic response to CD154 gene therapy for CLL.

The present study leads to several important questions about the role of STAT1 activation in the response to CD154 gene therapy and other biological therapeutic approaches to CLL. The first concerns the mechanism by which STAT1 becomes activated in the CLL cells. We considered two possibilities, direct cell-cell contact or the presence of a soluble mediator. Because direct cell-cell contact did not cause STAT1 activation in vitro, it is unlikely that surface protein interactions could induce STAT signaling. By contrast, STAT1 tyrosine phosphorylation did occur after incubation of CLL cells with plasma from some patients, suggesting that a cytokine may be responsible for inducing STAT signal transduction. However, because plasma caused detectable STAT1 activation in the cells of only 2 of 7 patients, it is possible that a mechanism other than cell-cell contact or the production of a soluble mediator is responsible for STAT1 activation.

In support of a mechanism for STAT1 activation involving a soluble mediator, such as a cytokine, high levels of Trx1 type cytokines (IFN-γ, IL-12, and IL-6) were detected within 8–48 h in the plasma of patients after CD154 gene therapy (5). We and others have found that each of these cytokines is capable of activating STAT1 in CLL cells. Although each is a plausible extracellular ligand responsible for STAT1 activation after infusion with Ad-CD154 CLL cells, a notable feature of STAT activation in this system is its restriction to STAT1. This indicates that a cytokine that activates only STAT1 and no other STAT proteins, such as IFN-γ, is most likely the factor responsible for STAT1 activation. However, not all of the plasmas containing detectable IFN-γ levels induced STAT1 activation. The two other Trx1 type cytokines detected in the patients, IL-12 and IL-6, are known to activate other STATs in addition to STAT1. Although we focused on the Trx1 cytokines known to be elevated in patients undergoing treatment, the possibility that other cytokines, growth factors, or hormones, or a combination of these factors could be responsible for STAT1 activation exists as well. It is also notable that STAT1 phosphorylation after infusion of Ad-CD154-CLL cells is prolonged, in contrast to the transient phosphorylation seen with cytokine stimulation in vitro. Such prolonged phosphorylation could represent a confluence of factors occurring in vivo or could be the result of the lack of induction of suppressors of cytokine signaling proteins, which act as negative regulators of STAT proteins. The latter hypothesis is supported by the observation that suppressors of cytokine signaling-1 mRNA is down-regulated in CLL cells (16). Although the present studies were unable to determine which mechanism was responsible for STAT1 activation, identifying the triggers leading to STAT1 activation remains an important question.

Although the biological effect of STAT1 activation in patients receiving CD154 gene therapy remains unknown, STAT1 activation most likely leads to the transcriptional activation of important target genes. One hypothesis is that increased activation of STAT1 leads to apoptosis of CLL cells. STAT1 activation preceded the acute decrease in ALC in patients undergoing CD154 gene therapy, which has been attributed to leukemic cell apoptosis (5). Thus, activation of STAT1 is consistent with its playing a proapoptotic role in the elimination of the malignant B cells during the acute phase of a therapeutic response. STAT1 is known to be important for transducing proapoptotic signals by inducing proapoptotic regulatory genes, such as caspases, Fas ligand, and tumor necrosis factor-related apoptosis-inducing ligand (17–25). Furthermore, STAT1 can promote apoptosis by inhibiting nuclear factor κB, a critical mediator of CD154 signal transduction (26). Whether any of the apoptotic regulatory genes known to be regulated by STAT1 are modulated after CD154 gene therapy remains to be examined.

An additional question is whether STAT1 activation is directly responsible for the therapeutic response or whether it is a marker for response. Serine phosphorylation in the absence of tyrosine phosphorylation may lead to enhancement of transcriptional activation or enhanced cell survival of CLL cells (11). By inducing tyrosine phosphorylation of STAT1, CD154 gene therapy may alter the abnormal STAT signaling in CLL cells to provide a more physiological outcome. If STAT1 activation directly leads to transcriptional activation of genes of which the gene products promote leukemic cell death or increased antigenicity of the CLL cell, then STAT1 could be directly responsible for the clinical and immunological responses observed in the patients. Activating STAT1 by inducing its tyrosine phosphorylation may be a feasible therapeutic strategy for CLL.

Our findings suggest that there may be potential prognostic value in measuring STAT1 activation after CD154 gene therapy. STAT1 activation can be detected within 24 h in patients who have significant decreases in ALCs and increased levels of IFN-γ. Therefore, it may be a useful early biological marker for response to therapy. Because some patients may not respond to the gene therapy, it would be useful to identify those patients early in their course by monitoring STAT1 tyrosine phosphorylation so that their therapy for CLL could be modified. Benekli et al. (27) demonstrated recently that constitutive STAT3 activation is associated with short disease-free survival in AML, supporting the notion that measuring STAT1 activity can be valuable in predicting disease outcome. In follow-up to our retrospective analysis, a prospective study evaluating the prognostic value of STAT1 activation in future trials for CD154 gene therapy or other biological therapies will be useful.

In conclusion, we have demonstrated that CLL cells from patients undergoing immune-based therapy have activated STAT1 within 24 h of treatment. Moreover, the activation of STAT1 is associated with both clinical and immunological therapeutic responses. The trigger responsible for STAT1 activation and the effect of STAT1 activation on CLL biology remains to be elucidated. Nonetheless, the monitoring of activation of transcription factors in vivo in response to biological therapies may provide insight into the mechanisms of these approaches and may yield important prognostic and predictive information.

REFERENCES


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