

Cell-based STAT signalling

Optimization of cytokine signalling conditions in human erythroleukemic TF-1 cells

Introduction

The Janus kinase (JAK)/signal transducers and activators of transcription (STAT) signalling pathway is associated with many downstream cellular responses, including proliferation, differentiation and survival. In particular, the JAK/STAT pathway is central to the control of hematopoiesis and immune development, mediating the response of cells to many different cytokines. The pathway is activated when a cytokine binds to a cell surface receptor, causing receptor dimerization, inducing a series of events that ultimately lead to STAT protein phosphorylation, dimerization and translocation to the nucleus. Once in the nucleus, activated STAT proteins are responsible for the transcription of specific genes.

Here we focus on the detection of the phosphorylation of key endogenous STAT proteins, namely STAT1, STAT3 and STAT5, in human erythroleukemic TF-1 cells. We demonstrate that the phosphorylation of these STAT proteins in TF-1 cells provides a reliable marker of cytokine receptor signalling, using cytokine-mediated phospho-STAT1, phospho-STAT3, and phospho-STAT5, as markers of IFN γ , IL-6 and GM-CSF, respectively.

Materials used

TF-1 cells are available from ATCC. AlphaScreen® SureFire® phospho-STAT1, phospho-STAT3, and phospho-STAT5 assay kits are available from PerkinElmer. Western blotting detection reagents and secondary antibody-HRP conjugates are available from PerkinElmer. Tissue culture plasticware is from Nunc, and AlphaScreen® SureFire® assay plates (Proxiplates™ and Optiplates™) were from PerkinElmer. IgG-free BSA was from Jackson ImmunoResearch. JAK inhibitor I was from Calbiochem.

Results

Detection of STAT phosphorylation in TF-1 cells by Western blot

To determine if detection of receptor-mediated endogenous STAT phosphorylation could be used to monitor cytokine signalling in a homogeneous cell based assay format, signalling *via* a number of different endogenous cytokine receptors in human erythroleukemic TF-1 cells was examined. In particular, lysates prepared from T25 flasks of cells stimulated with IFN γ , IL-6 or GM-CSF was monitored using the phosphorylation of STAT1, STAT3 and STAT5 proteins, and assessed by standard Western blot techniques (Figure 1). For each of STAT1, STAT3 and STAT5, agonist-mediated phosphorylation was readily detectable.

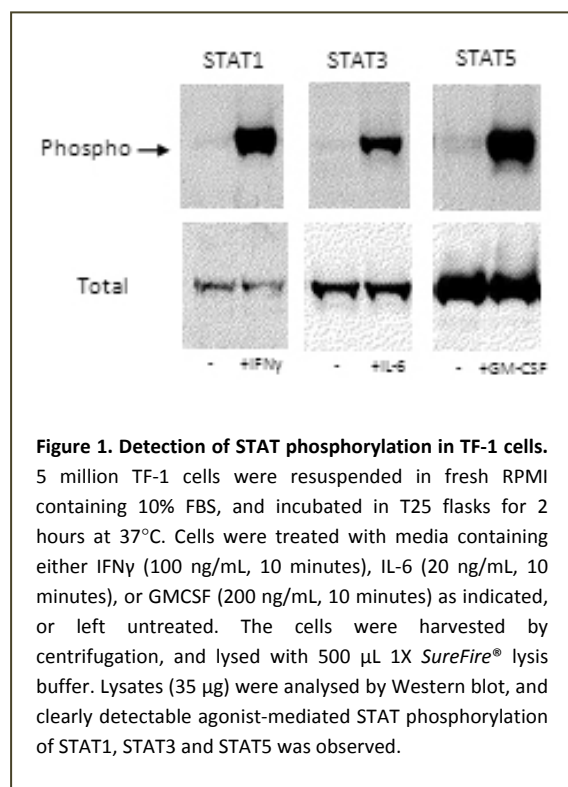


Figure 1. Detection of STAT phosphorylation in TF-1 cells. 5 million TF-1 cells were resuspended in fresh RPMI containing 10% FBS, and incubated in T25 flasks for 2 hours at 37°C. Cells were treated with media containing either IFN γ (100 ng/mL, 10 minutes), IL-6 (20 ng/mL, 10 minutes), or GMCSF (200 ng/mL, 10 minutes) as indicated, or left untreated. The cells were harvested by centrifugation, and lysed with 500 μ L 1X SureFire® lysis buffer. Lysates (35 μ g) were analysed by Western blot, and clearly detectable agonist-mediated STAT phosphorylation of STAT1, STAT3 and STAT5 was observed.

Detection of STAT phosphorylation in a microplate-based, homogeneous assay format

Optimal number of TF-1 cells

To establish if STAT phosphorylation could be adequately detected in a less laborious assay format, a microplate assay approach was developed. For this assay, cells are seeded, stimulated, lysed and assayed in a single microplate well. This approach affords the detection of a single analyte in a well, but with potential for high throughput of assay samples.

Firstly, an optimal cell number to use in single well assays was determined. TF-1 cells were seeded at a range of densities, from 10,000 cells per well, up to 50,000 cells per well into 384-well Optiplates™. The cells were stimulated with various agonists, and subsequently assayed for STAT phosphorylation (Figure 2). IFN γ -mediated STAT1 phosphorylation, IL-6-mediated STAT3 phosphorylation and GM-CSF-mediated STAT5 phosphorylation were all readily detectable in TF-1 cells at a concentration of as little as 10,000 cells per well, and good signal windows were achieved in the range of 10,000-30,000 cells per well. TF-1 cells seeded at a number in excess of 40,000 cells per well appeared to approach saturation of the assay.

Based on these results, for subsequent dose-response and dose inhibition experiments in 384-well plates, TF-1 cell-based assays were run using 30,000 cells per assay.

Dose stimulation of cytokine receptors in TF-1 cells

The performance of phospho-STAT detection assays as a marker of cytokine receptor activation in TF-1 cells was further assessed by responsiveness to various doses of the relevant agonists. Cells were seeded, stimulated, lysed and assayed in the same well, using the AlphaScreen® SureFire® assay system. TF-1 cells were stimulated with various doses of either IFN γ , IL-6 or GM-CSF for 10 minutes, then the cells were lysed, and analysed for p-STAT1, p-STAT3 or p-STAT5, respectively (Figure 3).

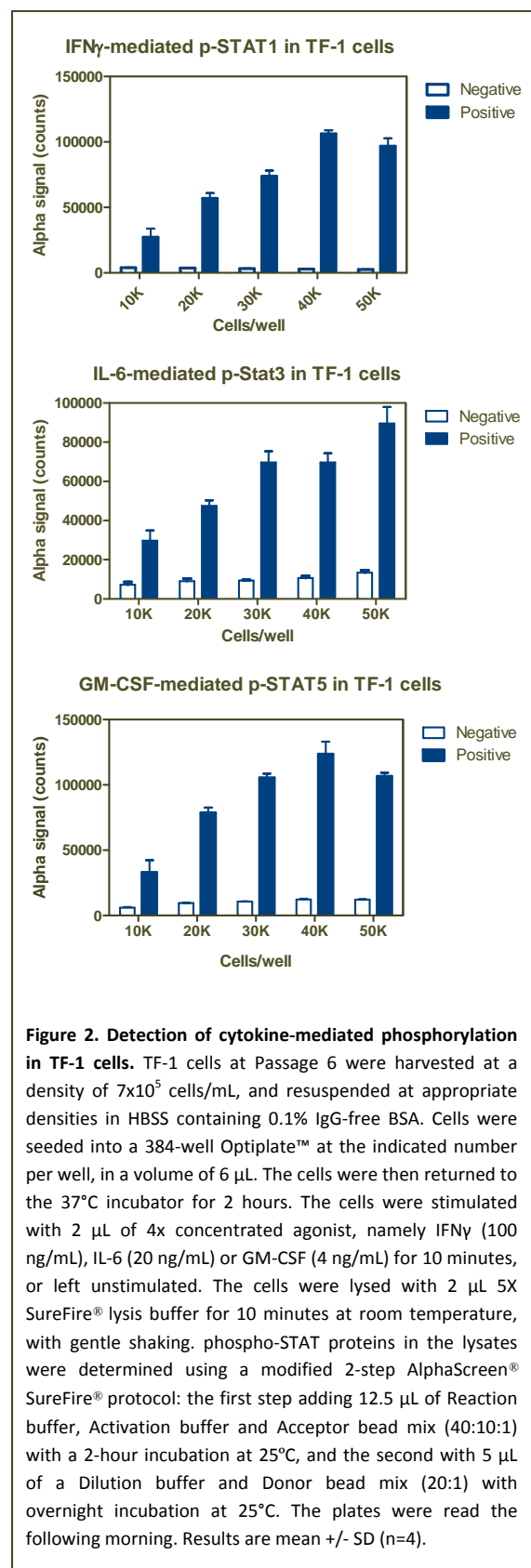
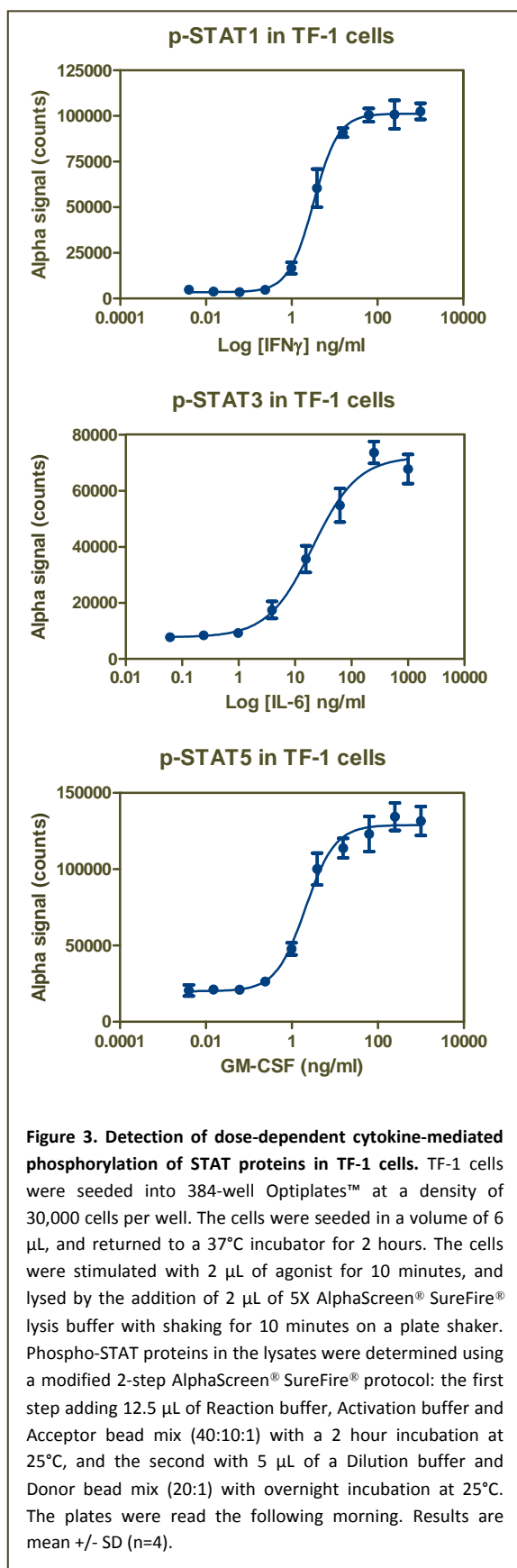


Figure 2. Detection of cytokine-mediated phosphorylation in TF-1 cells. TF-1 cells at Passage 6 were harvested at a density of 7×10^5 cells/mL, and resuspended at appropriate densities in HBSS containing 0.1% IgG-free BSA. Cells were seeded into a 384-well Optiplate™ at the indicated number per well, in a volume of 6 μ L. The cells were then returned to the 37°C incubator for 2 hours. The cells were stimulated with 2 μ L of 4x concentrated agonist, namely IFN γ (100 ng/mL), IL-6 (20 ng/mL) or GM-CSF (4 ng/mL) for 10 minutes, or left unstimulated. The cells were lysed with 2 μ L 5X SureFire® lysis buffer for 10 minutes at room temperature, with gentle shaking. phospho-STAT proteins in the lysates were determined using a modified 2-step AlphaScreen® SureFire® protocol: the first step adding 12.5 μ L of Reaction buffer, Activation buffer and Acceptor bead mix (40:10:1) with a 2-hour incubation at 25°C, and the second with 5 μ L of a Dilution buffer and Donor bead mix (20:1) with overnight incubation at 25°C. The plates were read the following morning. Results are mean \pm SD (n=4).

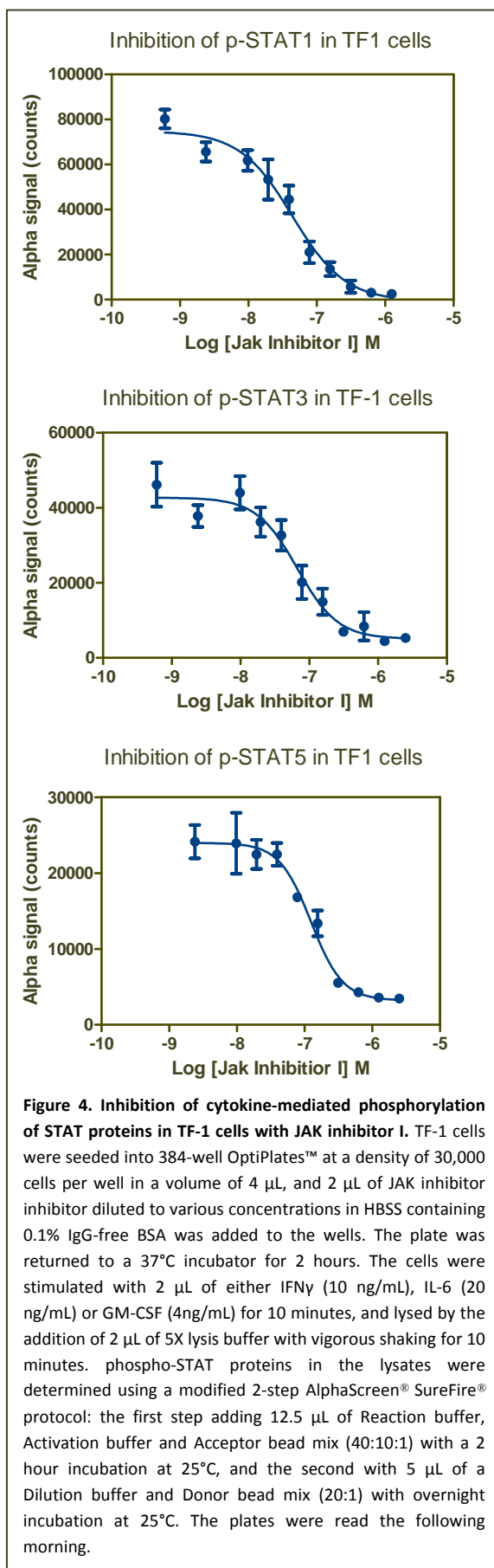


Dose-dependent phosphorylation of either STAT1, STAT3 or STAT5 was readily observed, indicating functional cytokine signalling pathways for each of the agonists selected.

Inhibition of cytokine-mediated STAT signalling in TF-1 cells

Finally, the cell-based assay system for cytokine receptor activation in TF-1 cells was assessed using the JAK inhibitor I. TF-1 cells were pre-treated with various doses of JAK inhibitor I for 2 hours, and stimulated with a sub maximal dose of either IFN γ , IL-6 or GM-CSF for 10 min, then the cells were lysed, and analysed for p-STAT1, p-STAT3 or p-STAT5, respectively (Figure 4).

The results showed a readily detectable, dose-dependent inhibition of phosphorylation of each of the cellular systems tested, with consistent IC₅₀ ranging from 5.3×10^{-8} M – 1.2×10^{-7} M. The results were generally consistent across receptor systems. Taken together, these results indicate that for the cell lines tested, endogenous STAT phosphorylation is a robust cell-based marker for activation of endogenous cytokine receptors in TF-1 cells.



Conclusions

We have demonstrated reliable detection of receptor-mediated signalling in TF-1 cells, and quite specific receptor-dependent patterns of STAT phosphorylation. These cellular assays can be tailored for both low-throughput or high-throughput assay applications, and afford quantitative data for the determination of receptor signalling mechanisms

Ordering Information

TF-1 cells are available from ATCC (www.atcc.org).

AlphaScreen® SureFire® cellular assay kits, available in 500pt, 10,000pt and 50,000pt pack sizes, are from PerkinElmer.

Catalog#

AlphaScreen SureFire Phospho-STAT1 (p-Tyr701)
TGRS1S500, TGRS1S10K and TGRS1S50K

AlphaScreen SureFire Phospho-STAT3 (p-Tyr705)
TGRS3S500, TGRS3S10K and TGRS3S50K

AlphaScreen SureFire Phospho-STAT5 A/B (p-Tyr694/699)
TGRS5S500, TGRS5S10K and TGRS5S50K

Note: These kits must be used in conjunction with the AlphaScreen protein A IgG detection kits.