

Simultaneous Measurement of STAT1, STAT3, and STAT5a/b Activations in Response to Interferons by Multiplex Bead Immunoassay

Mark Surby, Deanna Sarreal, Wei Zheng, Jimin Wang and Kevin Reagan

Invitrogen Corporation • 542 Flynn Road • Camarillo, CA 93012 • Correspondence to Mark.Surby@invitrogen.com

Introduction

Cytokines are a diverse group of low molecular weight proteins that mediate signals between cells. One subset of this protein family is the interferons (IFNs). IFNs are produced by the cells of the immune system in response to foreign agents such as viruses and tumor cells. In turn, these proteins assist the immune response by inhibiting viral replication, activating natural killer cells and increasing antigen presentation in lymphocytes. IFNs are classified according to cellular origin and extracellular receptor binding specificity. Type I IFNs include IFN- α and IFN- β . These IFNs most commonly induce anti-proliferative and anti-viral activity and bind to the type I IFN cell surface receptor. IFN- γ is a type II interferon which has weaker anti-viral activity than the type I IFNs, but more potent immunomodulatory properties. IFN- γ interacts with cells by binding to the type II IFN cell surface receptor. IFN receptor interactions by both types of IFNs leads to activation of intracellular kinases which transmit the signal to the cell nucleus through a series of phosphorylation events. The best characterized and commonly accepted IFN signaling pathway is the one in which members of the Janus kinase (JAK) family are activated upon IFN binding and then phosphorylate substrate proteins known as signal transducers and activators of transcription (STATs). The STAT family of proteins is comprised of STATs 1, 2, 3, 4, 5A, 5B, and 6. STAT family members are characterized by the presence of a DNA binding domain, an SH3 domain which mediates interaction with polyproline containing proteins, an SH2 domain which mediates interaction with phosphotyrosine containing proteins, and a C terminal transactivation (TAD) domain. STAT proteins control the transcription of specific genes in response to cytokine stimulation. The series of events leading to STAT activation arising from IFN stimulation is illustrated in **Figure 1**. **Figure 1A** shows extracellular IFN (represented by the blue triangle) prior to binding to its receptor. Upon binding (**Figure 1B**), the JAK kinases constitutively associated with the receptor subunits interact. The interacting JAK proteins transactivate one another by reciprocal tyrosine phosphorylation, and phosphorylate a tyrosine on two subunits contained in the receptor complex. These phosphorylated tyrosine residues provide paired docking sites for STAT via its SH2 domain (**Figure 1C**). STAT, recruited to the receptor complex, is then phosphorylated at a tyrosine residue by the JAKs. This tyrosine phosphorylation promotes STAT homo and heterodimerization mediated by reciprocal phosphotyrosine SH2 domain interaction and the dissociation of the dimers from the receptor complex (**Figure 1D**). The STAT dimer is translocated to the cell nucleus. Once in the nucleus, the STAT dimers complex with other nuclear proteins and regulate gene expression by binding to promoters or other response elements on DNA.

Given that the diverse cellular response to IFN exposure are mediated by a common pathway, it is likely that a variety of STAT homo- and heterodimer combinations are responsible for each individual phenotype through differential gene regulation. In order to elucidate a more complete picture of this mechanism, we have quantified the activation of three members of the STAT family (STAT1, STAT3 and STAT5) in a variety of cells that have been exposed to IFN- α , IFN- β or IFN- γ using both individual ELISAs and a multiplex bead immunoassay developed for the simultaneous detection of the activated proteins.

Experimental Outline

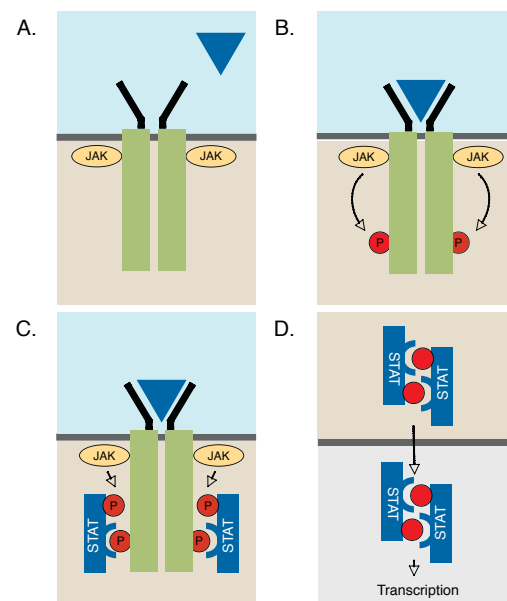
Four cell types were used for these experiments:

- K562 - human myelogenous leukemia cells
- Jurkat - human T lymphocyte cells
- TF-1 - human erythroleukemia cells
- THP-1 - human acute monocytic leukemia cells

Each cell line was grown in suspension to high density, serum starved for 24 hours and then aliquotted into 4 equal volumes. Each aliquot was treated with the following IFN concentrations for 10 minutes: 25 ng/ml IFN- α , 20 ng/ml IFN- β , 100 ng/ml IFN- γ or not treated (N.T.). Cell lysates were then made from each aliquot and the lysates brought to an equivalent concentration of 5 mg/ml total protein as determined by Bradford assay.

Each cell lysate was then analyzed for STAT1 [pY701], STAT3 [pY705] and STAT5a/b [pY694/699] levels in either individual ELISA assays or a multiplex bead immunoassay.

Figure 1—IFN mediated JAK-STAT signaling.



Results—Phospho Specific Multiplex Bead Immunoassay Quantitation of STAT

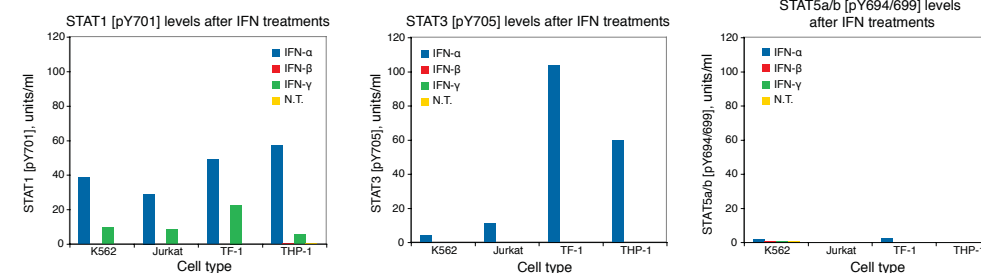


Figure 2—Differential STAT activation by cell type and IFN treatment. Phospho-specific multiplex bead immunoassay quantitation of STAT1, STAT3 and STAT5a/b activation in 4 different cell types. Cells were serum starved overnight and treated as indicated in the figure. Untreated cells were used as controls. Cell extracts were prepared from each treatment and 400 μ g/mL of each cell extract was analyzed using a single multiplex bead immunoassay.

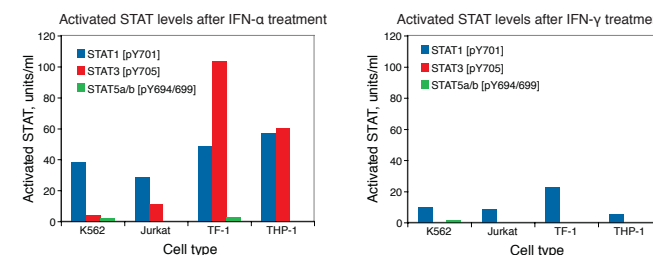


Figure 3—Treatment with IFN- α and IFN- γ show the highest degree of STAT activation. Phospho-specific multiplex bead immunoassay data re-plotted to illustrate patterns of STAT activation in each cell type treated with either IFN- α or IFN- γ .

Results—Phospho Specific ELISA Quantitation of STAT Activation

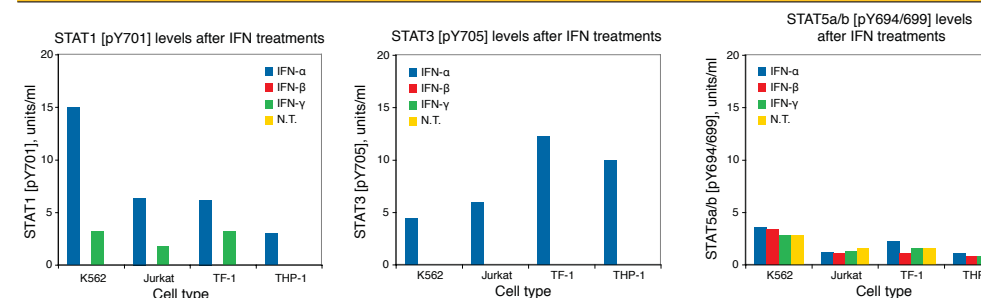


Figure 5—Differential STAT activation by cell type and IFN treatment. Phospho-specific ELISA quantitation of STAT1, STAT3 and STAT5a/b activation in 4 different cell types. Cells were serum starved overnight and treated as indicated in the figure. Untreated cells were used as controls. Cell extracts were prepared from each treatment and 500 μ g/mL of each cell extract was analyzed using three different ELISA kits.

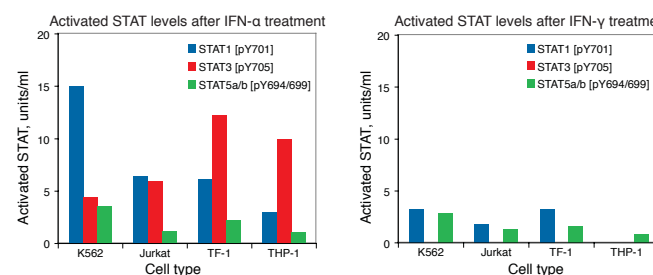
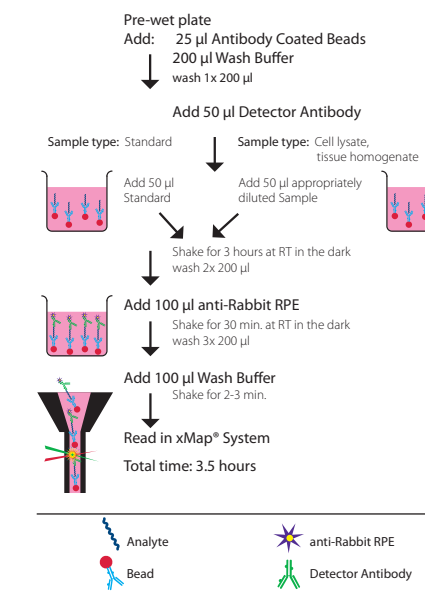


Figure 6—Treatment with IFN- α and IFN- γ show the highest degree of STAT activation. Phospho-specific ELISA data re-plotted to illustrate patterns of STAT activation in each cell type treated with either IFN- α or IFN- γ .

Figure 4—Multiplex Bead Immunoassay Summary



Discussion

All four different cell types showed significant activation of both STAT1 and STAT3 in response to IFN- α . Cellular response to IFN- γ treatment was mostly limited to STAT1 activation while treatment with IFN- β had no measurable effect. Levels of STAT5a/b activation remained low in all cells types and treatment conditions. What is most intriguing about all of these results is that the pattern of STAT activation was distinct for each different cell type.

Differential activation of STAT1 and STAT3 in the different cell types would seem to suggest the possibility for different STAT homo- and hetero-dimer populations in response to IFN- α and IFN- γ treatment. For example, significant activation of both STAT1 and STAT3 in TF-1 cells in response to IFN- α could suggest a greater population of STAT1/STAT3 hetero-dimers. This is in contrast to TF-1 cell response to IFN- γ , where only STAT1 is activated and STAT1 homo-dimers are the most likely transcriptional regulators.

We have performed quantitation of STAT activation levels using both phospho-specific ELISA assays and a multiplex bead immunoassay. While the patterns of STAT activation are similar between the two methods, there are some differences. ELISA measurements were 3- to 8-fold lower for STAT1 and STAT3 but were of similar magnitude to the multiplex bead immunoassay for STAT5a/b. In addition, the multiplex bead assay has significant advantages in terms of time and sample requirements.