Published in final edited form as:

J Immunol Methods. 2015 August ; 423: 3–11. doi:10.1016/j.jim.2015.03.018.

Simultaneous assessment of NF- κ B/p65 phosphorylation and nuclear localization using imaging flow cytometry

Orla Maguire^{a,*}, Kieran O'Loughlin^a, and Hans Minderman^a

^aLaboratory of Flow and Image Cytometry, Department of Pathology and Laboratory Medicine, Roswell Park Cancer Institute, Buffalo, NY

Abstract

Aberrant activity of Nuclear Factor- κ B (NF- κ B) is associated with many diseases and is therapeutically targeted. Post-translational modifications, particularly phosphorylation of the RELA/p65 sub-unit, are essential for cytoplasmic to nuclear localization of NF- κ B/p65 and initiation of transcription of downstream target genes. Immunoblot and phospho-flow cytometry has been used to study the relationship between phosphorylation motifs and NF- κ B activation and microscopic analysis of nuclear localization of p65 is also used as a parameter for activation. The labor intensive nature of these approaches commonly limits the number of sampling points or replicates. Recent insights in the relationship between p65 phosphorylation motifs and its nuclear localization indicate that these parameters have different significance and should not be used interchangeably. In this study, we demonstrate feasibility and reproducibility of studying the relationship between p65 phosphorylation and nuclear translocation using imaging flow cytometry (IFC). TNF α - or PMA/Ionomycin-induced phosphorylation of p65 at serine 529 in cell line models and healthy donor lymphocytes served as the experimental model. IFC analysis demonstrated that expression of phosphorylated serine 529 (P-p65^{s529}) increased rapidly following stimulation and that nuclear localization of P-p65^{s529} followed the nuclear localization pattern of total p65. However, in the presence of tacrolimus, P-p65^{s529} expression was inhibited without affecting nuclear localization of total p65. The data demonstrate the application of IFC to simultaneously assess phosphorylation of p65 and its cellular localization and the results obtained by this analysis corroborate current insights regarding the specific effect of tacrolimus on serine 529 phosphorylation.

Keywords

NF- κ B; p65; serine 529; phosphorylation; nuclear localization; imaging flow cytometry

Introduction

Phosphorylation of key proteins is an essential epigenetic event in cellular processes including those regulating proliferation, metabolism, and signal transduction (reviewed in (Banerjee and Chakravarti, 2011)). The study of phosphorylation in signal transduction

*Correspondence to: Orla Maguire, PhD, Flow and Image Cytometry, Roswell Park Cancer Institute, Cancer Cell Center, Elm and Carlton Streets, Buffalo, NY. Phone: (+1) 716-845-5890; Fax: (+1) 716-845-8806; orla.maguire@roswellpark.org.

events is especially important in pathways that are known to be dysregulated in disease states. Recent studies have defined a role for measuring the kinetics of phosphorylation events in not only the diagnosis/prognosis of diseases (Mackenzie et al., 2012; Sonnenblick et al., 2012; Woost et al., 2011), but also the efficacy of therapies, such as with the bcr/abl kinase inhibitor Imatinib, directed towards epigenetic alterations. (Druker et al., 2001; Hedley et al., 2008).

Nuclear Factor -kappa-light-chain-enhancer of B cells (NF- κ B) is a transcription factor activated by a variety of stimuli and is involved in key processes including proliferation, stress response, and cytokine production (Huang et al., 2010; Zheng et al., 2011). Its dysregulation is often implicated in inflammation, auto-immune disease, infections, and cancer (Ahmed, 2010; Brown et al., 2008; Nair et al., 2009; Wu and Zhou, 2010).

Activation of the NF- κ B transcription factor pathway requires a number of phosphorylation steps to allow transient cytoplasmic to nuclear localization (Oeckinghaus and Ghosh, 2009) and once nuclear, phosphorylated NF- κ B binds to promoter sequences of target genes to regulate their expression. Phosphorylation is a rapid and dynamic process, occurring within minutes of activation. Its rapid and transient nature makes it difficult to accurately measure phosphorylation states relevant to biological effects using techniques such as western blotting that due to their labor intensiveness or sample size requirements use limited sampling approaches.

Flow cytometry enables the determination of phosphorylation events in heterogeneous cell populations by co-staining cell populations with phospho-specific antibodies and antibodies for immunophenotyping (Chow et al., 2005; Lin et al., 2010; Perez et al., 2004). A caveat of conventional flow cytometry is the inability to determine the intracellular origin of a measured signal which may be especially relevant when studying signaling events.

In the NF- κ B signaling cascade, phosphorylation of p65 is required for nuclear translocation and transcriptional activation but different sites of phosphorylation have different significance in this regard. For example, phosphorylation at the serine 536 position is required for nuclear translocation while phosphorylation of serine 529 affects transcriptional activity and serine 276 is involved in co-activator recruitment at target gene promoters (Mattioli et al., 2004; Wang and Baldwin, 1998; Zhong et al., 1998). Thus, in the context of studying pharmacodynamic drug effects on NF- κ B activity, the relevance of using nuclear translocation of p65 as a parameter of response depends on the p65 phosphorylation sites that are affected.

Tacrolimus (TAC) is one of the cornerstones of contemporary immunosuppressive drug regimen. By inhibiting the activity of calcineurin, TAC inhibits the T cell receptor-mediated activation of the nuclear factor of activated T cells (NFAT), one of the key signaling pathways controlling the immune response on a cellular level. In addition to TAC's effect on calcineurin and the NFAT signaling pathway, TAC has recently been described to also affect the NF- κ B signaling pathway by inhibiting the phosphorylation of p65 at the serine 529 position (Vafadari et al., 2013). In order to demonstrate the applicability and relevance of IFC to simultaneously evaluate specific phosphorylation motifs and nuclear translocation,

TAC exposure of healthy donor T cells was used as the experimental model system with the expectation that TAC induced inhibition of p65 at the serine 529 position should be detectable without affecting nuclear translocation of total p65.

MATERIALS AND METHODS

All analysis files mentioned are available upon request.

Cell culture and NF- κ B activation

Cell lines were purchased from ATCC. Jurkat human lymphoblastic leukemia cells were maintained in RPMI-1640 media (Mediatech Inc., Manassas, VA) supplemented with 10% fetal bovine serum (PAA Laboratories Pty Ltd, Queensland, Australia), 2 mM L-glutamine, 20 U/mL penicillin, and 20 μ g/mL streptomycin (Mediatech Inc., Manassas, VA). HL-60 human promyelocytic leukemia cells were maintained in Iscoves media supplemented with 20% fetal bovine serum, L-glutamine, penicillin, and streptomycin, as above. Cell lines were maintained at exponential growth at 37°C in a fully humidified atmosphere of 5% CO₂ in air.

For NF- κ B activation, cell densities were adjusted to 1×10^6 cells/mL. Cells were exposed to 10 ng/mL Tumor Necrosis Factor alpha (TNF α) (Invitrogen, Carlsbad, CA) or 20 ng/mL PMA (Sigma, St. Louis, MO) / 1.5 μ M Ionomycin (Sigma, St. Louis, MO) for various time-points (see results) at 37°C, 5% CO₂ in air. Following activation, cells were fixed for 10 minutes in 4% methanol-free formaldehyde (FA) (Polysciences Inc, Warrington, PA) at room temperature and stained as outlined below.

Antibodies and staining

P-p65^{s529} was detected by direct labeling. Antibody was diluted in permeabilization wash buffer (PWB) consisting of 0.1% Triton-X-100 (EMD Biosciences, La Jolla, CA) in sterile 1 $\times$ phosphate buffered saline (PBS). The Mouse monoclonal P-p65^{s529}-AF488 antibody (BD Biosciences, San Diego, CA) was diluted 1:10 in PWB and incubated with cells at room temperature for 30 minutes. Antibodies were removed, cells washed with PWB, and resuspended in 100 μ L sterile 1 $\times$ PBS.

Total p65 and NFAT1 were detected by indirect labeling. Antibodies were diluted in PWB as above. The primary Rabbit polyclonal NF- κ B/p65 antibody (SantaCruz Biotechnology Inc, Santa Cruz, CA) was diluted 1:20 in PWB. Rabbit polyclonal NFAT1 antibody (Cell Signaling, Danvers, MA) was diluted 1:50 in PWB. Samples were incubated for 20 minutes at room temperature. Primary antibodies were removed, cells washed with PWB, and 1:200 dilution of secondary AF647 conjugated F(ab')₂ fragment donkey anti rabbit IgG antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was added and incubated at room temperature in the dark for 20 minutes. Secondary antibody was removed, cells washed with PWB, and resuspended in 100 μ L sterile 1 $\times$ PBS.

Just prior to data acquisition on the ImageStream, DAPI (Invitrogen, Carlsbad, CA) was added to all samples (0.5 μ g/mL final concentration), to stain the nucleus.

NF- κ B activation in healthy donor PBL *in vitro*

Healthy donor peripheral blood (PBL) was collected in accordance with a protocol approved by the Institutional Review Board at Roswell Park Cancer Institute. PBL was drawn in sodium heparinized tubes and allowed to 'rest' at room temp for at least 1 hour. 500 μ L PBL was used per sample and activation was initiated by adding either TNF α (100 ng/mL final concentration) or 50 ng/mL PMA (Sigma, St. Louis, MO) / 3.75 μ M Ionomycin (Sigma, St. Louis, MO) for various time-points (see results) at 37°C, 5% CO₂ in air. A control sample was included in the absence of activation stimulants. Following incubation, samples were immediately fixed using 20:1 ratio (9.5 mLs) of 4% FA diluted in 1 $\times$ PBS for 10 minutes at room temperature to preserve phosphorylation status. Following fixation, red blood cells were lysed using 9.5mLs 1 $\times$ BD Lyse Buffer according to the manufacturer's recommendations (BD Phosflow, BD Biosciences). Cells were then stained for NF- κ B/p65 and P-p65^{s529} as outlined above.

For Tacrolimus (TAC) inhibition, 500 μ L PBL was pre-treated with 10 nM TAC for 1.5 hrs at 37°C and then activated with PMA/Ionomycin as above with continued incubation with tacrolimus for 30 minutes. Control samples were incubated without stimulants and without TAC. Following activation, cells were fixed, red blood cells lysed, and stained for NFAT1 (as above).

ImageStream^X Acquisition

Data acquisition was performed on an imaging flow cytometer (Imagestream^X; Amnis/EMD Millipore, Seattle, WA) between years 2012-2014. Images acquired include a brightfield image (Channel 1 and 9; 430-480nm), FITC (Channel 2; 480-560nm), DAPI (Channel 7; 430-505nm), and AF647 (Channel 11; 660-740nm). AF488 was excited by a 488nm laser with a 100mW output, DAPI was excited by a 405nm laser with a 10mW output, and AF647 was excited by a 658nm laser with a 50mW output. The selected laser outputs prevented saturation of pixels in the relevant detection channels as monitored by the corresponding Raw Max Pixel features during acquisition. For each sample, bright field, P-p65^{s529}-AF488 with total p65-AF647 or NFAT1-AF647 and DAPI (nuclear stain) images were simultaneously collected for 20,000 events. Cell classifiers were set for the lower limit of size of the brightfield image to eliminate debris, the upper limit of size of the brightfield image to eliminate aggregates, a minimum intensity classifier on the DAPI channel to exclude non-cellular (DAPI negative) images, and, in healthy donor samples, an upper limit on scatter intensity to eliminate monocytes and granulocytes.

Compensation—In each experiment single color controls were stained for all fluorochromes and 500 events were collected with all relevant lasers on for each individual control. All channels were on, with brightfield LEDs and scatter laser off to accurately observe fluorescence overlap in all channels. Only those events exhibiting a positive signal in the channel of interest were collected (e.g., the P-p65^{s529}-AF488 control was positive in channel 2). Each single color control file was then merged to generate a compensation matrix (an example of which is shown in **Supplementary Figure 1**), and all sample files were processed with this matrix applied.

Data Analysis

Following compensation for spectral overlap based on single color controls, analysis was performed with IDEAS® software version 5.0 and individual cell images were created using IDEAS® software version 6.1 (Amnis Corp, Seattle, WA). Cell populations were hierarchically gated and an example of the gating strategy is shown in **Figure 1**. Single cells (A) that were in focus (B) and were positive for both DAPI and p65 (C) were selected as described previously (George et al., 2006; Maguire et al., 2011). All measurements of P-p65^{s529} were made from this parent total p65 population. In healthy donor samples, lymphocytes were gated based on positivity for p65 and scatter. After gates were applied, 70-80% of the total number of acquired images were incorporated into the final analysis. Expression levels of each factor in the entire cell are represented as 'Intensity'. Intensity in the IDEAS® software is calculated as the sum of the pixel values in the software-generated 'combined mask' minus the background pixel values (i.e., those not in the combined mask).

The spatial relationship between the transcription factors and nuclear images was measured using the 'Similarity' feature in the IDEAS® software, as described previously (George et al., 2006; Maguire et al., 2011). Briefly, a 'Morphology' mask is created to conform to the shape of the nuclear DAPI image, and a 'Similarity Score' (SS) feature is defined. The SS is a log-transformed Pearson's correlation coefficient between the pixel values of two image pairs, and provides a measure of the degree of nuclear localization of a factor by measuring the pixel intensity correlation between the factor-of-interest images and the DAPI images within the masked region. Cells with a low SS exhibit poor correlation between the images (corresponding with a predominant cytoplasmic distribution of the factor), whereas cells with a high SS exhibit positive correlation between the images (corresponding with a predominant nuclear distribution of the factor). The relative shift in this distribution between two populations (e.g., control versus treated cells) was calculated using the Fisher's Discriminant ratio (Rd value). Specifically, the mean SS of the test population (time-points > 0) minus the mean SS of the control population (time = 0) then divided by the sum of the standard deviations of both populations.

RESULTS

NF-κB activation by phosphorylation and nuclear localization can be measured by IFC

In order to determine whether activation of the P-p65^{s529} phosphorylation site could be measured by IFC simultaneously with total p65, the NF-κB/p65 signaling pathway was stimulated in the Jurkat cell line by exposure to PMA/Ionomycin for 30 minutes (a time-point previously determined to be optimal for total p65 nuclear translocation (Maguire et al., 2011)). As expected, total p65 expression did not change however the signal intensity relative to unstimulated cells, increased significantly for P-p65^{s529}. An example of typical histogram overlays observed for the intensity of both p65 and P-p65^{s529} is shown in **Figure 2A**. Nuclear localization of total p65 and P-p65^{s529} was also measured following activation for 30 minutes with PMA/Ionomycin (**Figure 2B**) using the similarity score (SS), a measure of the degree of change in similarity between the protein and nuclear images, detailed in the materials and methods. The median similarity score (Med SS) of the distributions is also provided. Representative images of the untreated cells (Unstim) show cytoplasmic

localization and PMA/Ionomycin-induced activation shows nuclear localization occurring for both total p65 and P-p65^{s529} (**Figure 2C**). The SS for each individual image is shown on its right in yellow.

Time kinetics of total p65 and P-p65^{s529} expression and nuclear localization in Jurkat T-cell line

In order to determine whether the activation kinetics of P-p65^{s529} measured by IFC compared to those of total p65, Jurkat cells were treated with either TNF α (**Figure 3A+C**) or PMA/Ionomycin (**Figure 3B+D**) for up to 60 minutes. Expression levels for P-p65^{s529} following TNF α activation significantly increased rapidly peaking at 10 minutes. As expected, total p65 expression levels did not change (**Figure 3A**). P-p65^{s529} expression then declined returning to baseline at around 40 minutes. Example images from 0, 10, 30, and 60 minutes samples are shown in **Supplementary Figure 2**). The kinetics of PMA/Ionomycin-induced p65 activation differed from that of TNF α in Jurkat cells. Expression of P-p65^{s529} was more gradual, peaking at 30 minutes, but similar to the TNF α data there is no expression change for total p65 (**Figure 3B**). The PMA/Ionomycin-induced activation remains significantly above baseline 60 minutes post-treatment.

In contrast, when measuring nuclear localization, TNF α induced both P-p65^{s529} and total p65 translocation significantly relative to time zero (untreated cells), with P-p65^{s529} peaking at 20 minutes, preceding a total p65 peak at 25 minutes (**Figure 3C**). At later time-points, the level of P-p65^{s529} in the nucleus returned close to untreated values, detailing the dynamic and short-lived nature of p65 phosphorylation events following activation by TNF α in this cell line, and mirroring the pattern of P-p65^{s529} expression. Total p65, however, is still significantly present in the nucleus after 60 minutes.

PMA/Ionomycin-activated kinetics also follow a different pattern than expression, where both total p65 and P-p65^{s529} localize in the nucleus more slowly, both peaking at 40 minutes (**Figure 3D**). Both factors then decline and have returned to cytoplasmic localization by 2 hours (data not shown). These results show that in this cell line, phosphorylation and nuclear localization measurements are comparable, thus confirming that nuclear translocation is an accurate alternative measure of activation at this step. The kinetics of activation by different stimulants however differs.

Similar results were observed when the same experiments were conducted on the promyelocytic leukemia HL-60. Expression of P-p65^{s529} increased following activation with TNF α and PMA/Ionomycin. Notably, IFC measured expression and nuclear translocation kinetics were more rapid in this cell line with activation causing a significant up-regulation at 2 minutes (**Suppl Figure 2**). Also of note in the HL-60 cell line, p65 nuclear localization could still be measured 4 hours post-treatment. The data thus demonstrate how the kinetics of NF- κ B activation using the same activating trigger, be it determined by expression of P-p65^{s529} or nuclear presence of p65, can differ between cell lines.

Time kinetics of total p65 and P-p65^{s529} expression and nuclear localization in healthy donor lymphocytes

In order to determine whether IFC can accurately measure P-p65^{s529} expression in lymphocytes *ex vivo*, healthy donor PBL samples were treated with PMA/Ionomycin for up to 60 minutes (**Figure 4**). NF- κ B activation was studied in the lymphocytes (gated based on p65 positivity and scatter properties). As expected, expression levels for total p65 following activation did not significantly rise above the untreated samples (**Figure 4A**, black line). Expression of P-p65^{s529} gradually increases, peaking at 20 minutes and then declining (**Figure 4A**, grey line). Nuclear translocation kinetics for total p65 showed a similar initial increase in nuclear localization peaking at 30 minutes (**Figure 4B**, black line). Nuclear translocation kinetics for P-p65^{s529} showed that P-p65^{s529} expression corresponds with its nuclear localization (**Figure 4B**, grey line). Interestingly, similar to the expression results, the P-p65^{s529} nuclear localization begins to decline after 20 minutes even when total p65 remains nuclear. Since this motif is involved in DNA binding to initiate the transcription of downstream target genes, its decline is likely necessary before total p65 leaves the nucleus.

IFC can measure the tacrolimus-induced inhibition of P-p65^{s529} phosphorylation where total p65 nuclear localization is unaffected

A recent study identified the immunosuppressive drug tacrolimus (TAC) as a novel inhibitor of p65 by direct inhibition of the P-p65^{s529} motif (Vafadari et al., 2013). This is unique since, to our knowledge, all other known inhibitors of P-p65^{s529} are in fact I κ B kinase inhibitors. To determine whether IFC could accurately measure this inhibition, peripheral blood mononuclear cells (PBMCs) were treated with 10 nM TAC, a concentration we have previously determined to be sufficient to completely inhibit TAC's canonical target, nuclear factor of activated transcription (NFAT1) (Maguire et al., 2013). Signaling was then stimulated by PMA/Ionomycin for 10 or 30 minutes and activation was studied in the lymphocytes (gated based on p65 positivity and scatter properties) (**Figure 5**). PMA/Ionomycin activated expression of P-p65^{s529} peaks at 10 minutes and is declining at 30 minutes in these samples (**Figure 5A**, grey line) TAC significantly inhibits the expression of P-p65^{s529} at both 10 minutes ($p=0.0112$) and 30 minutes ($p=0.004$) (black line). The observed inhibition of expression correlated with an inhibition of nuclear localization of P-p65^{s529} by 30 minutes (**Figure 5B**). Although this nuclear localization is not statistically significant it does trend down. Total p65 activation by PMA/Ionomycin is unaffected by addition of TAC (**Figure 5C**). As expected, activation by PMA/Ionomycin results in a complete shift of NFAT1 into the nucleus at both time-points (**Figure 5D**, grey line). Presence of 10 nM TAC completely inhibits PMA/Ionomycin induced nuclear translocation ($p<0.0001$) (black line).

Discussion

In this study the applicability of imaging flow cytometry (IFC) to simultaneously assess protein phosphorylation motifs and intracellular localization is demonstrated by determining the correlation between the phosphorylation of the NF- κ B subunit p65 on the serine 529 residue (P-p65^{s529}), the p65 nuclear localization, and the effects thereon of the immunosuppressant tacrolimus (TAC).

For the transcription factor NF- κ B, there are a number of sites on the p65 sub-unit that are phosphorylated by different kinases depending upon the activating trigger of the signaling pathway. Phosphorylated p65 serines include serine 276 (Vermeulen et al., 2003), serine 311 (Duran et al., 2003), serine 468 (Schwabe and Sakurai, 2005), serine 529 (Wang et al., 2000), serine 536 (Sakurai et al., 1999). The functional significance of the different phosphorylation sites with regards to NF- κ B transcriptional activity differ. The use of phospho-specific antibodies to detect expression levels of the various phosphorylated p65 motifs by flow cytometry has been used as a parameter of response to assess the activity of the NF- κ B pathway in relation to disease states or drug intervention (Hernandez Mde et al., 2011; Lin et al., 2013; Simard et al., 2014; Vafadari et al., 2013). Alternatively, since activation of NF- κ B is associated with relocation from the cytoplasm to nucleus, the nuclear localization of NF- κ B has also been used as a parameter for activation. Although both the phosphorylation and nuclear localization of p65 are necessary for transcriptional activity, neither one by itself is sufficient to warrant transcription. Therefore in absence of an NF- κ B transcription-dependent functional read out, it is important to assess both. The data demonstrate that in absence of TAC, the patterns of p65^{S529} phosphorylation are similar to those of its nuclear translocation but that the time kinetics of both can differ between cell lines and healthy donors and by different stimulants of activation. It is further demonstrated that TAC affects the serine 529 phosphorylation but without an effect on nuclear translocation of total p65. In the Jurkat cell line, the serine 529 phosphorylation and the nuclear translocation of total p65 are comparable. Serine 529 is located on the transactivation domain 1 (TA1) on p65 and is involved in the activation of transcription of NF- κ B downstream target genes by promoter binding (Madrid et al., 2001; Wang and Baldwin, 1998; Wang et al., 2000). In a more tumorigenic cell line, the promyelocytic leukemic cell line, HL-60, the pattern of phosphorylation kinetics was quite different from those observed in Jurkat cells. Again, the phosphorylation event and nuclear translocation of total p65 occurred very rapidly within 2 minutes of TNF α activation. Although in this cell line both total p65 and P-p65^{S529} continued to be localized in the nucleus after 4 hours treatment, at 3 hours the P-p65^{S529} expression levels had returned close to baseline levels possibly due to the more oncogenic nature of NF- κ B in malignant cells, where activation is commonly sustained (Pickering et al., 2007).

Similar to the cell line models, activation of NF- κ B with PMA/Ionomycin, in healthy donor lymphocytes, resulted in comparable P-p65^{S529} phosphorylation event and total p65 translocation. As expected, expression of total p65 did not change but expression of phosphorylated Ser529 increased. At 30 minutes the total p65 nuclear localization peaked, whereas P-p65^{S529} expression and nuclear localization was declining. Since serine 529 is involved in initiation of target gene transcription this decline may be necessary before p65 can return to the cytoplasm. Both remained present (above baseline) after 60 minutes. Increasingly, NF- κ B is considered a therapeutic target particularly in diseases of immune function, and hematological malignancies (Breccia and Alimena, 2010; Haddad and Abdel-Karim, 2011). Since the time kinetics of phosphorylation events and nuclear translocation of total p65 can differ, the timing of sampling is critical in interpretation of detection of phospho-specific p65 species as a parameter of NF- κ B signaling activity. It is also important to note that the choice of phosphorylation motif to be studied should be carefully considered

depending on the model system and the cellular events studied. This study, and other recent literature, indicates that specific motifs are activated differently to differentially regulate their downstream targets. Serine 529, but not serine 536, is essential in stem cell differentiation (Yang et al., 2010), and serine 276, specifically, is involved in monocyte-derived macrophage survival (Wang et al., 2011). Serine 311 phosphorylation is abrogated in anergic T cells, and inhibits p65-induced IFN γ production without affecting serine 536 phosphorylation (Clavijo and Frauwirth, 2012). This study demonstrates that TAC can inhibit P-p65^{S529} phosphorylation but not the nuclear localization of p65. Interestingly, although expression decreases, it remains nuclear. Our previous studies have already shown that TAC inhibits IFN γ production (Maguire et al., 2013). Further studies into the pattern of this serine 529 inhibition and its specific effects on IFN γ and other downstream targets are ongoing in our lab.

In summary, the data demonstrate the application of IFC to simultaneously assess a phosphorylation motif of p65 and its relevance to the cellular localization of p65. The results obtained by IFC analysis corroborate the current insights derived from analysis with other platforms regarding the relation between the effect of TAC on serine 529 phosphorylation and the nuclear localization of p65. The IFC approach is much less labor intensive and time consuming than alternative analysis approaches like immunoblot or microscopy. Additionally, IFC provides the intracellular localization parameter which is highly relevant in case of the activity of transcription factors such as NF- κ B which is unattainable by conventional flow cytometry analysis. The observed variable relationships between specific phosphorylation motifs and nuclear translocation events as a parameter of activation is likely not a phenomenon specific to NF- κ B, but could be applicable also to other transcription factor pathways, including the MAPK (Desterke et al., 2011; Fischer et al., 2010) and Jak/STAT pathways (Diaz et al., 2011; Lesterhuis et al., 2011).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors wish to acknowledge Dr. Sherree Friend (Amnis Corp) for continued application support with regards to ImageStream data acquisition and analysis.

Research sponsored by NIH R33CA126667 (H.M.) and by the NCI Cancer Center Support Grant to the Roswell Park Cancer Institute (CA016056)

References

- Ahmed A. Prognostic and therapeutic role of nuclear factor-kappa B (NF-kappaB) in breast cancer. *Journal of Ayub Medical College, Abbottabad : JAMC.* 2010; 22:218–221. [PubMed: 22338460]
- Banerjee T, Chakravarti D. A peek into the complex realm of histone phosphorylation. *Molecular and cellular biology.* 2011; 31:4858–4873. [PubMed: 22006017]
- Breccia M, Alimena G. NF-kappaB as a potential therapeutic target in myelodysplastic syndromes and acute myeloid leukemia. *Expert opinion on therapeutic targets.* 2010; 14:1157–1176. [PubMed: 20858024]

- Brown KD, Claudio E, Siebenlist U. The roles of the classical and alternative nuclear factor-kappaB pathways: potential implications for autoimmunity and rheumatoid arthritis. *Arthritis research & therapy*. 2008; 10:212. [PubMed: 18771589]
- Chow S, Hedley D, Grom P, Magari R, Jacobberger JW, Shankey TV. Whole blood fixation and permeabilization protocol with red blood cell lysis for flow cytometry of intracellular phosphorylated epitopes in leukocyte subpopulations. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2005; 67:4–17. [PubMed: 16080188]
- Clavijo PE, Frauwirth KA. Anergic CD8+ T lymphocytes have impaired NF- kappaB activation with defects in p65 phosphorylation and acetylation. *J Immunol*. 2012; 188:1213–1221. [PubMed: 22205033]
- Desterke C, Bilhou-Nabera C, Guerton B, Martinaud C, Tonetti C, Clay D, Guglielmelli P, Vannucchi A, Bordessoule D, Hasselbalch H, et al. FLT3-mediated p38-MAPK activation participates in the control of megakaryopoiesis in primary myelofibrosis. *Cancer research*. 2011; 71:2901–2915. [PubMed: 21487043]
- Diaz T, Navarro A, Ferrer G, Gel B, Gaya A, Artells R, Bellosillo B, Garcia-Garcia M, Serrano S, Martinez A, et al. Lestaurtinib inhibition of the Jak/STAT signaling pathway in hodgkin lymphoma inhibits proliferation and induces apoptosis. *PloS one*. 2011; 6:e18856. [PubMed: 21533094]
- Druker BJ, Sawyers CL, Kantarjian H, Resta DJ, Reese SF, Ford JM, Capdeville R, Talpaz M. Activity of a specific inhibitor of the BCR-ABL tyrosine kinase in the blast crisis of chronic myeloid leukemia and acute lymphoblastic leukemia with the Philadelphia chromosome. *The New England journal of medicine*. 2001; 344:1038–1042. [PubMed: 11287973]
- Duran A, Diaz-Meco MT, Moscat J. Essential role of RelA Ser311 phosphorylation by zetaPKC in NF-kappaB transcriptional activation. *The EMBO journal*. 2003; 22:3910–3918. [PubMed: 12881425]
- Fischer H, Lutay N, Ragnarsdottir B, Yadav M, Jonsson K, Urbano A, Al Hadad A, Ramisch S, Storm P, Dobrindt U, et al. Pathogen specific, IRF3-dependent signaling and innate resistance to human kidney infection. *PLoS pathogens*. 2010; 6:e1001109. [PubMed: 20886096]
- George TC, Fanning SL, Fitzgerald-Bocarsly P, Medeiros RB, Highfill S, Shimizu Y, Hall BE, Frost K, Basiji D, Ortyrn WE, et al. Quantitative measurement of nuclear translocation events using similarity analysis of multispectral cellular images obtained in flow. *Journal of immunological methods*. 2006; 311:117–129. [PubMed: 16563425]
- Haddad JJ, Abdel-Karim NE. NF-kappaB cellular and molecular regulatory mechanisms and pathways: therapeutic pattern or pseudoregulation? *Cellular immunology*. 2011; 271:5–14. [PubMed: 21777910]
- Hedley DW, Chow S, Goolsby C, Shankey TV. Pharmacodynamic monitoring of molecular-targeted agents in the peripheral blood of leukemia patients using flow cytometry. *Toxicologic pathology*. 2008; 36:133–139. [PubMed: 18337231]
- Hernandez Mde O, Fulco Tde O, Pinheiro RO, Pereira Rde M, Redner P, Sarno EN, Lopes UG, Sampaio EP. Thalidomide modulates Mycobacterium leprae-induced NF-kappaB pathway and lower cytokine response. *Eur J Pharmacol*. 2011; 670:272–279. [PubMed: 21925494]
- Huang B, Yang XD, Lamb A, Chen LF. Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway. *Cellular signalling*. 2010; 22:1282–1290. [PubMed: 20363318]
- Lesterhuis WJ, Punt CJ, Hato SV, Eleveld-Trancikova D, Jansen BJ, Nierkens S, Schreiber G, de Boer A, Van Herpen CM, Kaanders JH, et al. Platinum-based drugs disrupt STAT6-mediated suppression of immune responses against cancer in humans and mice. *The Journal of clinical investigation*. 2011; 121:3100–3108. [PubMed: 21765211]
- Lin CC, Huang WL, Su WP, Chen HH, Lai WW, Yan JJ, Su WC. Single cell phospho-specific flow cytometry can detect dynamic changes of phospho-Stat1 level in lung cancer cells. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2010; 77:1008–1019. [PubMed: 20814891]
- Lin G, Field JJ, Yu JC, Ken R, Neuberger D, Nathan DG, Linden J. NF-kappaB is activated in CD4+ iNKT cells by sickle cell disease and mediates rapid induction of adenosine A2A receptors. *PloS one*. 2013; 8:e74664. [PubMed: 24124453]

- Mackenzie L, McCall P, Hatzieremia S, Catlow J, Adams C, McArdle P, Seywright M, Tanahill C, Paul A, Underwood M, et al. Nuclear factor kappaB predicts poor outcome in patients with hormone-naïve prostate cancer with high nuclear androgen receptor. *Human pathology*. 2012
- Madrid LV, Mayo MW, Reuther JY, Baldwin AS Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *The Journal of biological chemistry*. 2001; 276:18934–18940. [PubMed: 11259436]
- Maguire O, Collins C, O'Loughlin K, Miecznikowski J, Minderman H. Quantifying nuclear p65 as a parameter for NF-kappaB activation: Correlation between ImageStream cytometry, microscopy, and Western blot. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2011; 79:461–469. [PubMed: 21520400]
- Maguire O, Tornatore KM, O'Loughlin KL, Venuto RC, Minderman H. Nuclear translocation of nuclear factor of activated T cells (NFAT) as a quantitative pharmacodynamic parameter for tacrolimus. *Cytometry Part A : the journal of the International Society for Analytical Cytology*. 2013; 83:1096–1104. [PubMed: 24136923]
- Mattioli I, Sebald A, Bucher C, Charles RP, Nakano H, Doi T, Kracht M, Schmitz ML. Transient and selective NF-kappa B p65 serine 536 phosphorylation induced by T cell costimulation is mediated by I kappa B kinase beta and controls the kinetics of p65 nuclear import. *J Immunol*. 2004; 172:6336–6344. [PubMed: 15128824]
- Nair RP, Duffin KC, Helms C, Ding J, Stuart PE, Goldgar D, Gudjonsson JE, Li Y, Tejasvi T, Feng BJ, et al. Genome-wide scan reveals association of psoriasis with IL-23 and NF-kappaB pathways. *Nature genetics*. 2009; 41:199–204. [PubMed: 19169254]
- Oeckinghaus A, Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harbor perspectives in biology*. 2009; 1:a000034. [PubMed: 20066092]
- Perez OD, Krutzik PO, Nolan GP. Flow cytometric analysis of kinase signaling cascades. *Methods Mol Biol*. 2004; 263:67–94. [PubMed: 14976361]
- Pickering BM, de Mel S, Lee M, Howell M, Habens F, Dallman CL, Neville LA, Potter KN, Mann J, Mann DA, et al. Pharmacological inhibitors of NF-kappaB accelerate apoptosis in chronic lymphocytic leukaemia cells. *Oncogene*. 2007; 26:1166–1177. [PubMed: 16924235]
- Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IkappaB kinases phosphorylate NF-kappaB p65 subunit on serine 536 in the transactivation domain. *The Journal of biological chemistry*. 1999; 274:30353–30356. [PubMed: 10521409]
- Schwabe RF, Sakurai H. IKKbeta phosphorylates p65 at S468 in transactivation domain 2. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*. 2005; 19:1758–1760. [PubMed: 16046471]
- Simard C, Cloutier M, Neron S. Feasibility study: phosphospecific flow cytometry enabling rapid functional analysis of bone marrow samples from patients with multiple myeloma. *Cytometry B Clin Cytom*. 2014; 86:139–144. [PubMed: 24243860]
- Sonnenblick A, Shriki A, Galun E, Axelrod JH, Daum H, Rottenberg Y, Hamburger T, Mali B, Peretz T. Tissue microarray-based study of patients with lymph node-positive breast cancer shows tyrosine phosphorylation of signal transducer and activator of transcription 3 (tyrosine705-STAT3) is a marker of good prognosis. *Clinical & translational oncology : official publication of the Federation of Spanish Oncology Societies and of the National Cancer Institute of Mexico*. 2012; 14:232–236.
- Vafadari R, Kraaijeveld R, Weimar W, Baan CC. Tacrolimus inhibits NF-kappaB activation in peripheral human T cells. *PloS one*. 2013; 8:e60784. [PubMed: 23573283]
- Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G. Transcriptional activation of the NF-kappaB p65 subunit by mitogen-and stress-activated protein kinase-1 (MSK1). *The EMBO journal*. 2003; 22:1313–1324. [PubMed: 12628924]
- Wang D, Baldwin AS Jr. Activation of nuclear factor-kappaB-dependent transcription by tumor necrosis factor-alpha is mediated through phosphorylation of RelA/p65 on serine 529. *The Journal of biological chemistry*. 1998; 273:29411–29416. [PubMed: 9792644]

- Wang D, Westerheide SD, Hanson JL, Baldwin AS Jr. Tumor necrosis factor alpha-induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. *The Journal of biological chemistry*. 2000; 275:32592–32597. [PubMed: 10938077]
- Wang Y, Mo X, Piper MG, Wang H, Parinandi NL, Guttridge D, Marsh CB. M-CSF induces monocyte survival by activating NF-kappaB p65 phosphorylation at Ser276 via protein kinase C. *PloS one*. 2011; 6:e28081. [PubMed: 22216091]
- Woost PG, Solchaga LA, Meyerson HJ, Shankey TV, Goolsby CL, Jacobberger JW. High-resolution kinetics of cytokine signaling in human CD34/CD117-positive cells in unfractionated bone marrow. *Blood*. 2011; 117:e131–141. [PubMed: 21330471]
- Wu Y, Zhou BP. TNF-alpha/NF-kappaB/Snail pathway in cancer cell migration and invasion. *British journal of cancer*. 2010; 102:639–644. [PubMed: 20087353]
- Yang C, Atkinson SP, Vilella F, Lloret M, Armstrong L, Mann DA, Lako M. Opposing putative roles for canonical and noncanonical NFkappaB signaling on the survival, proliferation, and differentiation potential of human embryonic stem cells. *Stem Cells*. 2010; 28:1970–1980. [PubMed: 20882529]
- Zheng C, Yin Q, Wu H. Structural studies of NF-kappaB signaling. *Cell research*. 2011; 21:183–195. [PubMed: 21135870]
- Zhong H, Voll RE, Ghosh S. Phosphorylation of NF-kappa B p65 by PKA stimulates transcriptional activity by promoting a novel bivalent interaction with the coactivator CBP/p300. *Molecular cell*. 1998; 1:661–671. [PubMed: 9660950]

Highlights

- IFC can simultaneously measure phosphorylation and nuclear translocation of p65
- Tacrolimus inhibits P-p65^{s529} phosphorylation but not nuclear translocation of p65

Figure 1. Hierarchical gating and analysis strategy used to determine transcription factor expression and nuclear translocation

Single cells are discriminated from debris and cell aggregates based on area and aspect ratio of the brightfield image (**A**). Of those cells, events which are in focus are selected on the basis of a high value of a contrast parameter (gradient RMS of the brightfield image) (**B**). Single, in focus cells that positive for both a nuclear signal and total p65 signal are then selected (**C**). At this point cell line samples would progress to step **E** (see below). In healthy donor PBMC samples gating for lymphocytes was performed based on p65 positivity and scatter properties (**D**). A software-generated ‘morphology mask’ based on the nuclear image (**E**) is then applied to the ‘Similarity’ feature comparing the transcription factor and nuclear images. A distribution of cells with varying ‘similarity scores’ is then graphed (**F**), and the mean and standard deviation recorded to measure the ‘Rd value’. The median similarity score (Median SS) of the example distribution is also shown.

Figure 2. P-p65^{s529} expression and nuclear translocation of p65 as parameters of response to PMA/Ionomycin-induced NF-κB activation

Activation of p65 was triggered in the Jurkat cell line using PMA/Ionomycin for 30 minutes. Expression level, measured by intensity, increases above unstimulated levels for P-p65^{s529} but not total p65 as shown by histogram overlays for both total p65 (left graph) and P-p65^{s529} (right graph) (A). Translocation into the nucleus, measured by similarity score (SS), significantly increases for both total p65 and P-p65^{s529} when cells are stimulated with PMA/Ionomycin (B). Median similarity score is also shown for each population. Representative images of nuclear translocation in both untreated (Unstim) and cells treated with PMA/Ionomycin are shown (C). From left to right in each panel, BF = brightfield images of each cell, followed by the nuclear image (blue) total p65 (red) and P-p65^{s529} (green) images, and the far right column is the merged image of the nucleus with total p65 and P-p65^{s529}. The SS for total p65 and P-p65^{s529} is shown on the right of the individual image (yellow).

Figure 3. Time kinetics of activation-induced total p65 and P-p65^{s529} expression and nuclear localization in Jurkat cells

Jurkat cells were stimulated with TNF α (A+C) or PMA/Ionomycin for various time durations up to 1 hour (B+D). Expression level is measured as fold change of intensity relative to untreated zero time-point set to 1. With TNF α , expression level of P-p65^{s529} peaks at 10 minutes, with no change in total p65 (A). Nuclear translocation, measured by Rd Value, of P-p65^{s529} peaks at 20 minutes and total p65 translocation peaks at 25 minutes (B). With PMA/Ionomycin, expression level of P-p65^{s529} peaks at 30 minutes, with no change in p65 (C). Nuclear translocation of both total p65 and P-p65^{s529} peaks at 40 minutes (D). Results are representative of 3 independent experiments, error bars represent SEM (*p<0.05, **p<0.01, ***p<0.001, Students t test).

Figure 4. Time kinetics of PMA/Ionomycin-induced p65 and P-p65^{s529} expression and nuclear localization in healthy donor lymphocytes

Whole blood samples from hematologically healthy donors were stimulated with PMA/Ionomycin for various time durations up to 1 hour. Expression level is measured as fold change of intensity relative to untreated zero time-point set to 1. No change in expression level of p65 is observed, however expression level of P-p65^{s529} peaks at 20 minutes (A). Nuclear translocation, measured by the Rd value, of total p65 peaks at 30 minutes whereas P-p65^{s529} peaks at 15 minutes. Both remain in the nucleus at 60 minutes. Results are representative of 3 independent experiments, error bars represent SEM (*p<0.05, **p<0.01, Students t test).

Figure 5. Tacrolimus (TAC)-inhibits PMA/Ionomycin-induced P-p65^{s529} expression without affecting nuclear translocation of p65 in healthy donor T-cells

Peripheral blood mononuclear cells from hematologically healthy donors were stimulated with PMA/Ionomycin for 10 minutes or 30 minutes. Expression level of P-p65^{s529}, measured as fold change of intensity relative to untreated zero time-point set to 1, is inhibited at both 10 and 30 minutes post PMA/Ionomycin treatment (A). Nuclear translocation of P-p65^{s529} is inhibited at 30 minutes (B). Nuclear translocation of total p65 is unaffected by TAC at either time-point (C). As a control, the nuclear translocation of the transcription factor NFAT1 is completely inhibited by TAC (D). Results are representative of 3 independent experiments, error bars represent SEM (*p<0.05, **p<0.01, ****p<0.0001, Students t test)