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TNF-induced MAP kinase activation oscillates in time

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ABSTRACT

Oscillations in the activation of multiple signaling pathways have never been shown before. Our results presented in the previous accompanying paper showed that TNF induces highly dynamic oscillations in mRNA production in ~13% of the mouse genome. Here, we further analyze the TNF time-series microarray data and find that multiple signaling components downstream of the TNF receptor undergo oscillations. Prior studies implicate IκBα and A-20 as the only oscillatory components in the TNF signaling cascade. We find however, that other components, such as TRAF1, displayed oscillations. This suggested the possibility that all signaling output from the TNF receptor may be oscillatory in nature. Indeed, we show that TNF triggers oscillations in the phosphorylation of three MAP kinases, as well as p65. Because Baltimore and colleagues had proposed that NF-κB drives the oscillatory nature of the IκBα/NF-κB feedback loop, we studied the effects of an NF-κB super-repressor on oscillations in MAPK phosphorylation; we find that the super-repressor altered the amplitude and frequency of MAP kinase phosphorylation, but failed to abolish oscillations. These results attest to a role for NF-κB as a modulator, but not the sole determinant of cyclical activation of signal transduction pathways. These results, together with those of the two accompanying papers, constitute a new paradigm through which cells orchestrate signaling molecules to produce highly dynamic physiological processes such as gene transcription and protein secretion. In view of the discovery that multiple phosphorylation pathways display dynamic oscillations, time-resolved, instead of static, measurements of kinase phosphorylation should become the experimental norm.

Oscillations in the phosphorylation of signaling molecules have been shown in only in three situations, Hes-1, p53, and IκB. In these three examples, the protein oscillations serve to govern feedback loops of the proteins involved. NF-κB regulates numerous genes involved in cellular stress responses, cell growth, cell survival, and apoptosis. The oscillatory nature of NF-κB activation is governed by a negative feedback system where NF-κB proteins induce the expression of an inhibitor of their nuclear translocation, IκBα [1]. The NF-κB–IκBα complex is exported into the cytoplasm preventing further NF-κB action and thus creating a feedback loop [1].

In the preceeding accompanying paper, we showed that the inflammatory cytokine TNF triggered oscillations in mRNA production for >5000 genes (~13% of the genome). Moreover, we showed that these continuous oscillations are not unique to TNF, but that TNF superfamily member cytokines such as RANK-L were also capable of inducing oscillations in gene transcription. We found that each cytokine had a distinctive mRNA induction profile over time, despite initial similarities in gene induction.

The results from the accompanying paper also showed that mRNA oscillation frequencies were as low as every 50 min. This suggests that mRNA production is highly dynamic. We hypothesize that this dynamic nature may be the result of rapidly changing events further upstream. Specifically, we hypothesize that multiple signaling components downstream of the TNF receptor may display dynamic oscillations in activation. In this paper, we first analyze the microarray data of the preceeding paper and find that several components of the TNF signaling pathway, such as TRAF1, undergo oscillations in their mRNA production. We then use phospho-flow cytometry to show that TNF triggers oscillations in the phosphorylation of MAP kinases. Through the use of an IκB super-repressor, we show that the oscillations in MAP kinase phosphorylation are independent of, but modulated by NF-κB. In the following paper, we show that the oscillations combine to recruit transcription factors to promoters in a cyclical fashion.

Results

The preceding paper showed that TNF induces dynamic changes in gene expression with a frequency that has never been appreciated before. To investigate the mechanistic basis of these dynamic changes, we performed a pathway analysis on the two clusters

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containing early and late oscillator genes. KEGG pathway analysis, using a *z*-score cut-off of 2.0 to indicate significance, identified several functional signaling pathways within the two clusters. Several key genes appeared in multiple KEGG pathways. For the early oscillators, these included IκBα, Ras-GRP1, TRAF-1, MALT1, SOCS3, PTK2 (FAK1), and TNF itself (Table 1). For the late oscillators, statistically significant association were noted with NF-κB p100, IAP, p15, and Fas (Table 1).

Fig. 1 shows a composite diagram of TNF receptor signaling containing the molecules annotated as early or late oscillators. It is obvious that while NF-κB components are most downstream, other oscillators, such as Ras-GRP1, TRAF1, and MALT1 are upstream. This oscillator profile prompted us to question whether the phosphorylation of NF-κB components, such as p65, and distal MAP kinases, such as JNK1/2, ERK, and p38, was also oscillatory. Using a 96-well phospho-ELISA plate, we first measured phosphorylated p65 and JNK every 10 min for 0.5 h and every 30 min thereafter for 8 h following TNF application to primary murine macrophages. We found that the phosphorylation of both p65 and JNK oscillated over time, *albeit* with broad, somewhat poorly defined peaks (Fig. 2A and B).

To validate these results in another cell type, to obtain a better time resolution, and for further mechanistic experiments, intracellular phospho-flow cytometry was performed on a Jurkat cell line. The phosphorylation of p65, JNK, and p38 was oscillatory for up to 8 h (Fig. 2C–E). The first phosphorylation peak for p65, JNK, p38, and ERK1/2 overlapped, after which ERK1/2 phosphorylation became rapidly attenuated (Fig. 2F). Moreover, the phosphorylation profiles for p38 and p65 were in unison (Fig. 2G). JNK phosphorylation also oscillated more rapidly than p65 or p38 phosphorylation in the first 3 h. At around 4 h, p65, p38, and JNK phosphorylation became simultaneously aligned (Fig. 2G). This time point is significant in that it coincides with induction of late oscillator genes (see preceeding paper), but whether or not the coincidental phosphorylation of this trio mediates gene induction is evaluated in the following paper.

Because all three MAP kinases, p38, JNK, and ERK1/2, displayed oscillations in phosphorylation, we theorized that a feedback mechanism within the early TNF signaling cascade likely mediated these oscillations. Using IκB null cells, Baltimore and co-workers first identified cyclical production of IκBα as a negative feedback loop to cyclically inhibit NF-κB activation [1]. We have verified the cyclical phosphorylation of p65, but find that this can also occur in wild type cells, not solely in the mutant cells they have used. Using KEGG pathway analysis, we have further identified both TNF and TRAF1 as early oscillators identical to IκBα (Fig. 1). TRAF1 is a known inhibitor of TNF receptor signaling [2], and is directly upstream of both p38 and JNK, but is more distantly related to ERK (Fig. 1). Because TNF and TRAF1 are both NF-κB-dependent genes, we chose to examine whether abrogating NF-κB signaling could affect the phosphorylation of the two MAP kinases JNK and ERK1/2.

JNK and ERK phosphorylation were thus examined in Jurkat cells over-expressing an IκB super-repressor construct, which specifically suppresses p65 translocation. Fig. 2I and J show comparisons of JNK and ERK phosphorylation in IκB-dominant negative (IκB-DN) overexpressing (solid lines) and wild type (broken lines) cells. Of note is that the oscillations in the phosphorylation of both MAP kinases persisted despite abrogation of NF-κB signaling. However, the amplitude of JNK phosphorylation was significantly increased and the oscillation frequency altered in IκB-DN cells (Fig. 2I). Additionally, the frequency of ERK1/2 phosphorylation was converted from monophasic to oscillatory (Fig. 2J).

Fig. 2H shows that in IκB-DN cells, ERK1/2, and JNK oscillations were in unison for the first 3 h following TNF. Furthermore, ERK1/2 did not show any appreciable phosphorylation after 4 h (Fig. 2K), whereas JNK continued to oscillate with a frequency and peak width distinct from that of empty vector transfectants (Fig. 2I). Together, these data show that while NF-κB is not necessary for the genesis of MAP kinase oscillations, it regulates their amplitude and frequency.

Discussion

The results show for the first time that TNF induces robust and rapid oscillations in the phosphorylation of the distal kinases JNK, ERK1/2, and p38, as well as p65. The pattern of these oscillations suggests that there are several key time points where all signaling pathways are simultaneously activated. Through the use of the NF-κB super-repressor, the amplitude, and frequency of these oscillations is regulated, but is not generated by the previously demonstrated cyclic activation of NF-κB. Although decoding the mechanistic basis of these oscillations will represent a challenge, two key issues arise.

Table 1
Analysis of gene sub-clusters reveals affected pathways and multiple oscillatory components in the TNF signaling cascade

KEGG pathway	*z*-score	Oscillatory components
Small cell lung cancer		
Early oscillators	4.04	IκBα, TRAF1, Prostaglandin-endoperoxide synthase 2, FAK1
Late oscillators	3.47	NF-κB p100, p15, INK4b, IAP
T cell receptor signaling		
Early oscillators	3.82	IκBα, RasGRP1,TNF, and MALT 1
Late oscillators	1.14	NF-κB p100
Adipocytokine signaling		
Early oscillators	3.27	IκBα, SOCS3, and TNF
Late oscillators	1.57	NF-κB p100 and FACS (acetyl-CoA synthetase)
Apoptosis		
Early oscillators	2.93	IκBα, IL-1, and TNF
Late oscillators	2.47	NF-κB p100, IAP, and Fas
TGF-beta signaling		
Early oscillators	2.93	Smurf1, ID3, and TNF
Late oscillators	0.21	p15 INK48
Diabetes pathways		
Early oscillators	2.65–2.77	SOCS3, IL-1, and TNF
Late oscillators	2.25–3/52	Fas, MHCII, HNF6, and Nr5a2

Primary macrophages were subject to microarray analysis. Early and late oscillator genes are defined in the preceding paper. KEGG pathway analysis was used to derive *z*-scores, and statistically significant oscillators with a *z*-score >2 were identified.