SAND2008-6254C

CHARACTERIZATION OF THE TLR4-MEDIATED RESPONSE TO *Y. PESTIS* AND *E. COLI* LPS IN MURINE MACROPHAGES

Catherine Branda, Steven Branda, Elizabeth Carles, Bryan Carson, Jaclyn Murton, Jens Poschet, Anup Singh
Sandia National Labs
7011 East Ave.
Livermore, CA, 94550

ABSTRACT

The toll-like receptors (TLRs) are key sentries of the innate immune system, specifically recognizing molecular signatures presented by pathogens; for example, TLR4 is activated by lipopolysaccharides (LPS) derived from bacterial membranes. Our current interest is in the role of TLR4 in enabling macrophages to combat infection by the bacterial agents *Francisella tularensis* (tularemia) and *Yersinia pestis* (the plague). We have developed new reagents for analysis of TLR4 pathway activation and dynamics, as well as a novel experimental device capable of high-throughput, high-resolution interrogation of single cells. We have applied these new tools to study TLR4-mediated responses to LPS, including that derived from *F. tularensis* and *Y. pestis*, and have focused primarily on the phosphorylation and translocation dynamics of RelA and the MAPKs. This work has improved our understanding of the molecular mechanisms underlying innate immunity, laying the groundwork for development of next-generation diagnostics and therapeutics in service of biodefense.

INTRODUCTION

The toll-like receptors (TLRs) are required for the mammalian immune response to microbes. TLR4, for example, is the receptor for lipopolysaccharide (LPS), a major component of the outer membrane of gram-negative bacteria. Stimulation of the TLR4 receptor complex by LPS initiates a signal transduction cascade culminating in production and secretion of cytokines. A central node in this signal transduction cascade is the transcription factor nuclear factor (NF)-κB, normally maintained in the cytoplasm by the IκB inhibitors. Activation of the TLR4 pathway results in targeted IκB proteolysis, freeing NF-κB to move to the nucleus where it activates target gene expression. Transcriptional targets of NF-kB include the cytokine tumor necrosis factor (TNF), and the IκB inhibitor, whose resynthesis redistributes NF-kB back to the cytoplasm. Sustained cellular exposure to TNF has been shown to induce NF-kB oscillations in and out of the nucleus (1, 2), though the biological significance of this oscillatory behavior has been debated (1, 2, 5). We have demonstrated, for the first time, that NF-kB oscillatory behavior is observed in murine macrophages in response to LPS, and have developed a cell line that enables us to monitor, simultaneously, NF-kB localization and TNF transcription. Using live cell imaging and QT-PCR, we show that NF-kB oscillatory behavior is correlated with oscillatory expression of known NF-kB transcriptional targets.

RESULTS

FIGURE 1A: RelA-GFP oscillates in and out of the nucleus in murine macrophages in response to sustained stimulation with 100nM *E. coli* LPS. Each shape in the graph corresponds to a particular cell, monitored over time.

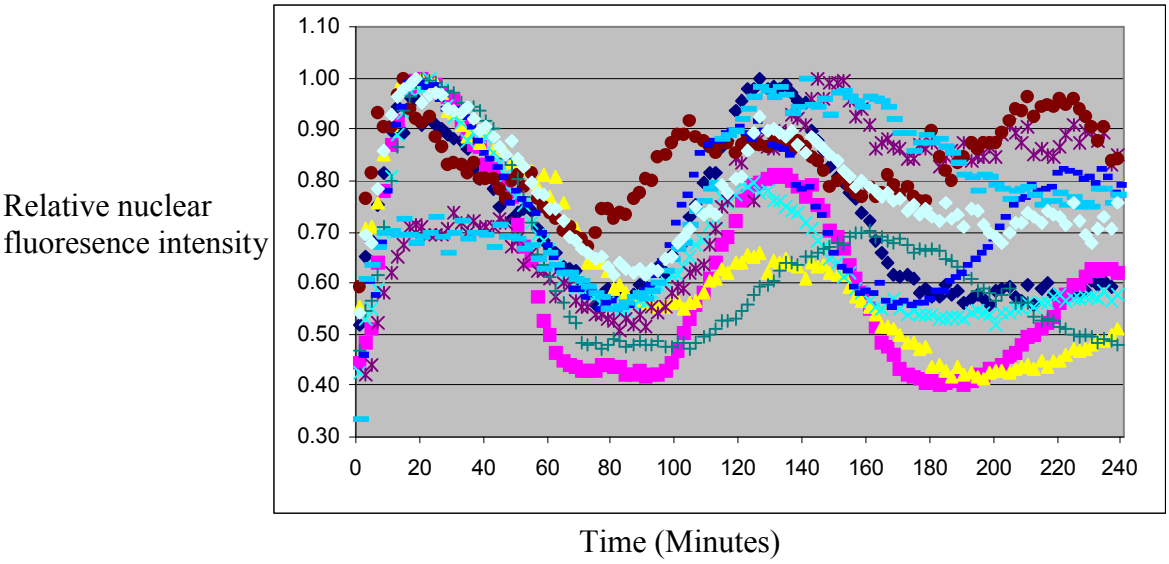


FIGURE 1B: RelA-GFP oscillates in and out of the nucleus in response to 1nM Y. pestis 21^{o}, but not 1nM Y. pestis 37^{o}

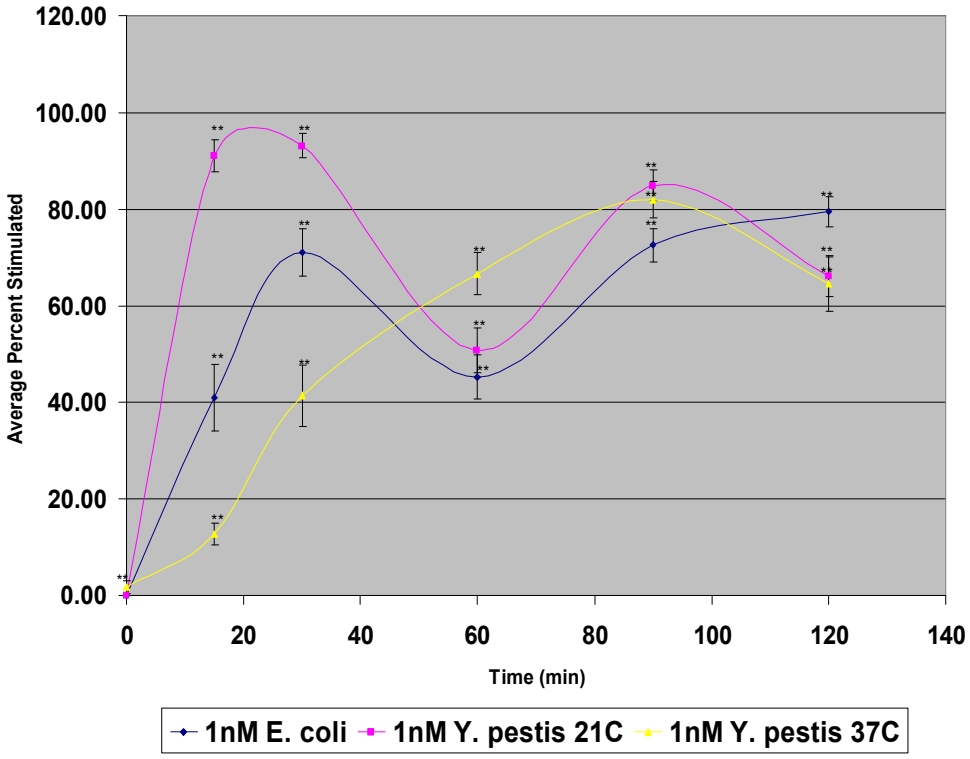


FIGURE 2: Oscillation in mRNA production of NF-kB transcriptional targets in murine macrophage stimulated with 1mM E. coli LPS

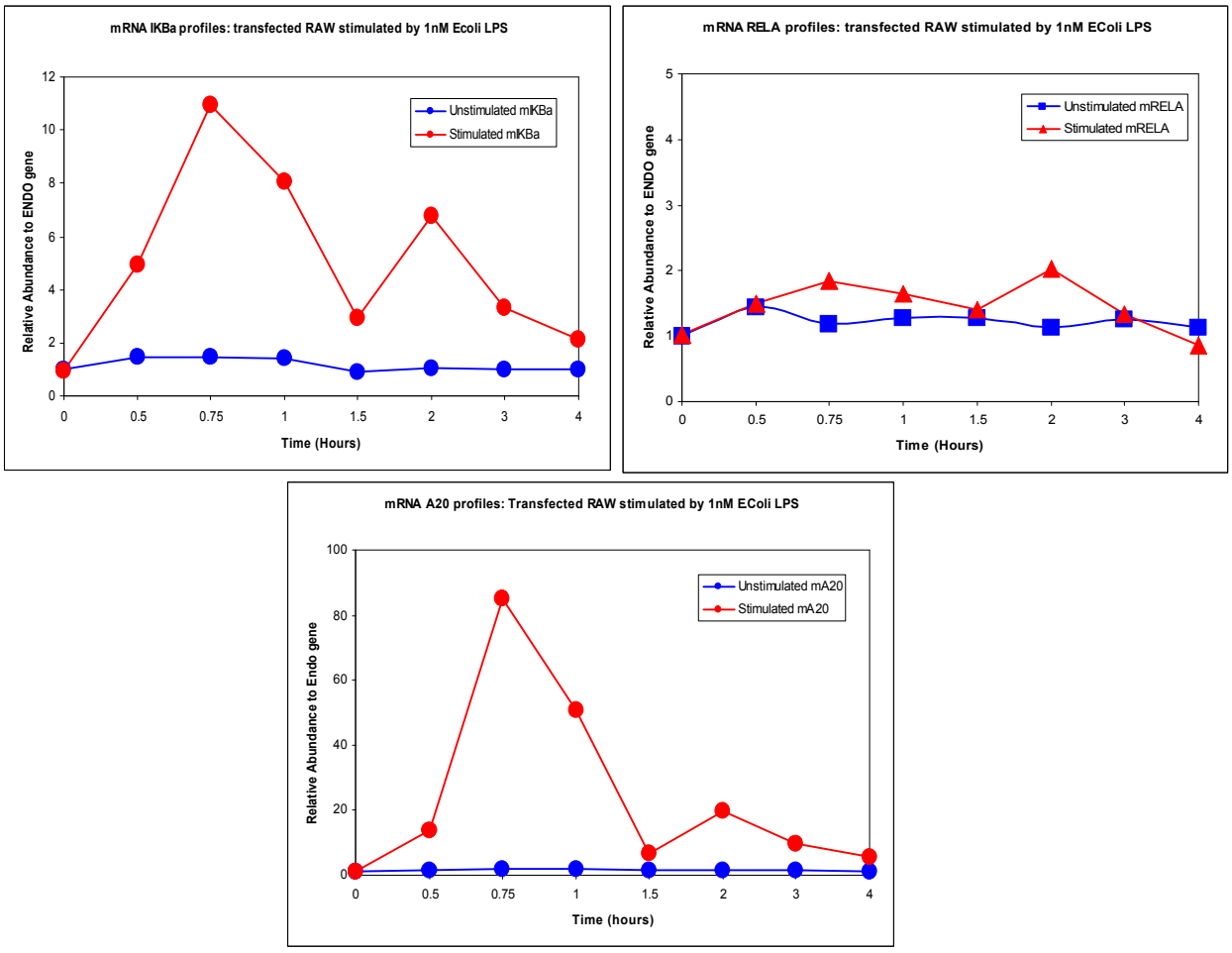
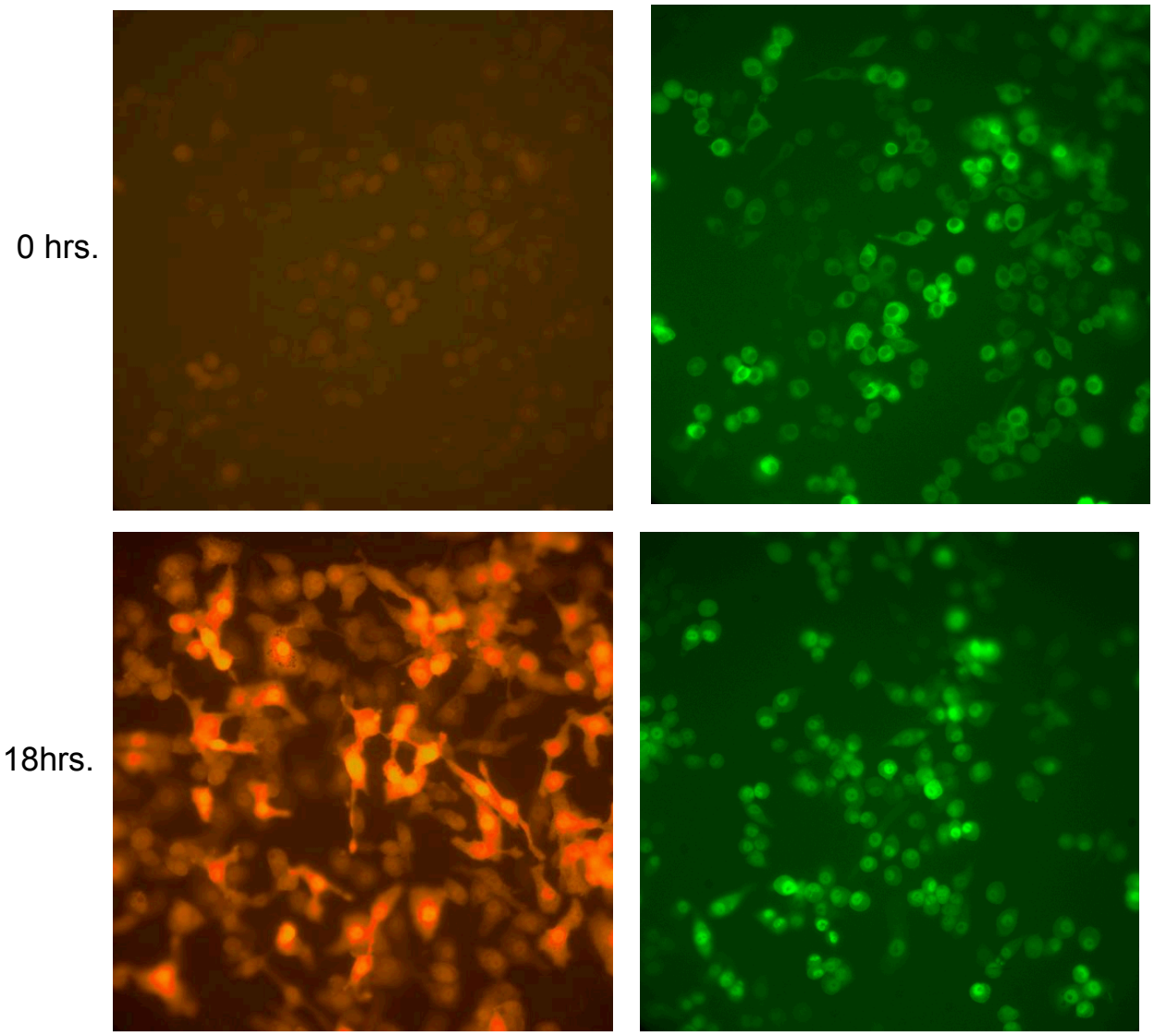


FIGURE 3: Fluorescence expression in stable murine macrophage cell line expressing TNFa-mCherry-PEST transcriptional reporter and RelA-GFP at 0 and 18 hours post stimulation with 1nM E. coli LPS



METHODS

CELL LINES:

The $p_{\beta Actin}$-EGFP-RelA construct was derived from pECFP-F-RelA, a kind gift from Dr. Allan Brasier (University of Texas Medical Branch). ECFP was replaced with EGFP between the Age1 and BsrG1 sites, and the cytomegalovirus (CMV) promoter was replaced with a minimal 106bp human βActin promoter (3) cloned between the Ase1 and Nhe1 sites to reduce average expression levels. The plasmid pBA-GFP-RelA was linearized and used to transfect RAW264.7 murine macrophage-like cells (ATCC) by Nucleofection (Amaxa Biosystems). Transfected cells were grown for 12 days in the presence of G418, and a clone stably expressing GFP-RelA was isolated and named RG16.

The $P_{TNF\alpha}$-mCherry-PEST transcriptional reporter was generated by subcloning a 1.2Kb TNFα promoter fragment (a kind gift from Dr. Yon Rojanasakul, W. Virginia University School of Pharmacy) between the NheI and HIII sites of a modified pcDNA3.1/Hygro(+) vector (Invitrogen), from which the CMV promoter had been excised. Subsequently, coding sequence for mCherry (4) fused to a PEST sequence (derived from pd1GFP, BD Biosciences) was inserted between the HIII and XhoI sites, to generate $P_{TNF\alpha}$-mCherry-PEST. A stable cell line expressing both the GFP-RelA fusion and the TNFα-mCherry-PEST transcriptional reporter was generated by transfection of the construct into RG16 cells and subsequent selection in the presence of both G418 and Hygromycin.

For subsequent experiments, cells were grown on untreated polystyrene dishes in DMEM supplemented with 10% fetal bovine serum (ATCC), 2mM L-glutamine, 1mM sodium pyruvate, 1x MEM nonessential amino acids, 20mM HEPES, 100 I.U./ml penicillin, and 100µg/ml streptomycin (all supplements from Mediatech) at 37°C with 5% CO_2.

IMAGING:

The intensities of nuclei were extracted and normalized using CellProfiler™, cell positions were tracked using MatLab™, and intensity and time curves were exported with Excel.

QPCR:

Total RNA was isolated from either RAW264.7 or RG16 murine macrophages stimulated with 0nM, 1nM or 100nM *E.coli* LPS at the following timepoints: 0, 30, 45, 60, 90, 120, 180, and 240min. The total RNA isolation was repeated on subsequent days to obtain two biological replicas for each experimental condition. TotalRNA extraction was performed using Qiashredder, RNAeasy, and DNase on-column kits from Qiagen. RNA integrity was tested using a Bioanalyzer. QPCR experiments were performed using the ABI 7500. Two technical replicas were analyzed for each of two biological replicas.

CONCLUSIONS

Stimulation of the TLR4 receptor complex by LPS initiates a signal transduction cascade culminating in production and secretion of cytokines. A central node in this signal transduction cascade is the transcription factor NF-κB, shown previously to oscillate in and out of the nucleus in response to TNF stimulation. How NF-κB translocation dynamics regulate gene expression is still poorly understood, yet this understanding is critical for its effective targeting in diagnostic and therapeutic applications. To address this knowledge gap, we have developed tools to enable correlation of NF-κB localization with TNFα production in living cells, and have combined live cell imaging with QT-PCR studies to study how NF-κB oscillatory behavior is correlated with gene expression in murine macrophages. We have used these tools to demonstrate that NF-kB

oscillatory behavior is observed in murine macrophages in response to LPS, and that this dynamic expression is correlated with oscillatory expression of known NF-kB transcriptional targets.

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