

Kinetics of Intracellular Nitric Oxide Production and Cytotoxicity in Lipopolysaccharide and Interferon-gamma Induced Macrophage Activation

KimVan Tran and Kamala Tyagarajan

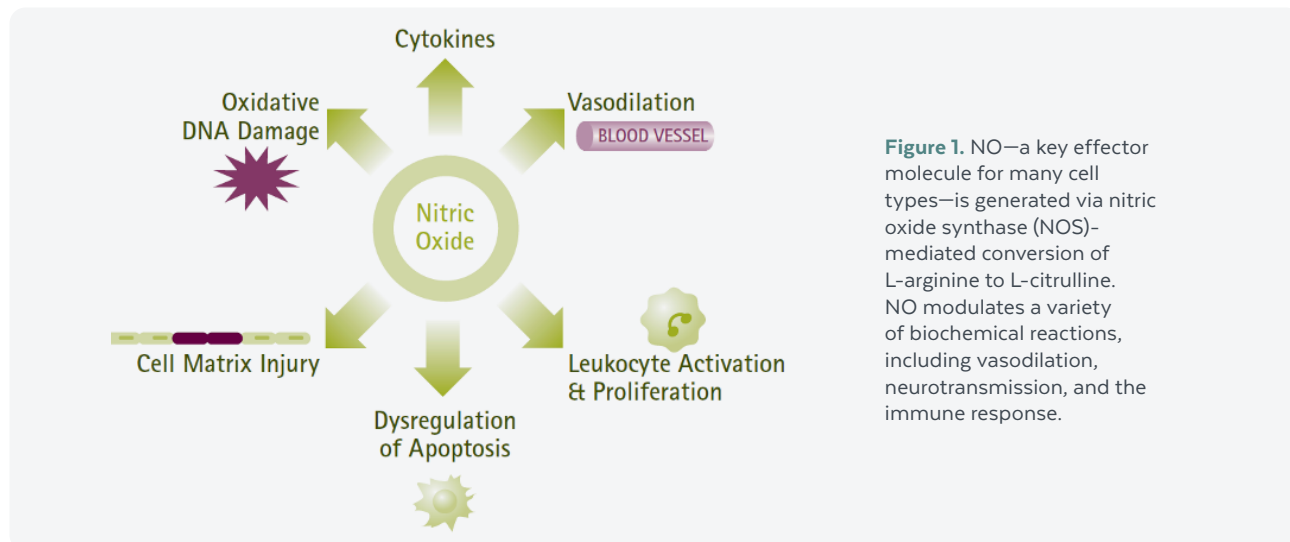
Introduction

Nitric oxide (NO) is a small reactive gaseous molecule that is generated in a wide variety of cells—notably, neurons and cells of the immune system. Because it diffuses readily across cellular membranes, NO has the potential for transcellular signaling activity as well as intracellular effects. As an effector molecule, it is important for many key processes, including cellular proliferation, apoptosis, and cancer progression, where it has been implicated in tumorigenesis, tumor killing, and metastasis in a concentration-dependent manner (**Figure 1**).

In the context of the innate immune response, NO functions as a bactericidal and tumoricidal mediator. It is well-established that bacterial lipopolysaccharides (LPS) and the proinflammatory cytokine interferon-gamma (IFN γ) are both potent inducers of NO production in macrophages, where NO functions as a key effector molecule in the host defense response to microbial pathogens. In addition to its effector immune functions, endogenous NO production also contributes to the modulation of cell cycle, apoptosis, and cell death.

In this study, we systematically evaluated the mechanisms and kinetics of NO production, as well as the subsequent cytotoxicity following LPS and IFN γ treatment of the murine macrophage cell line RAW264.7. Using the Guava[®] Muse[®] Cell Analyzer—a simplified, microcapillary flow cytometry detection instrument with optimized fluorescence-based assays—stimulated macrophages were assessed for NO production. Concurrent apoptotic activity in these cells was measured using Muse[®] assays for multiple apoptosis pathways.

Figure 1. Cellular Functions and Consequences of Nitric Oxide (NO) Production



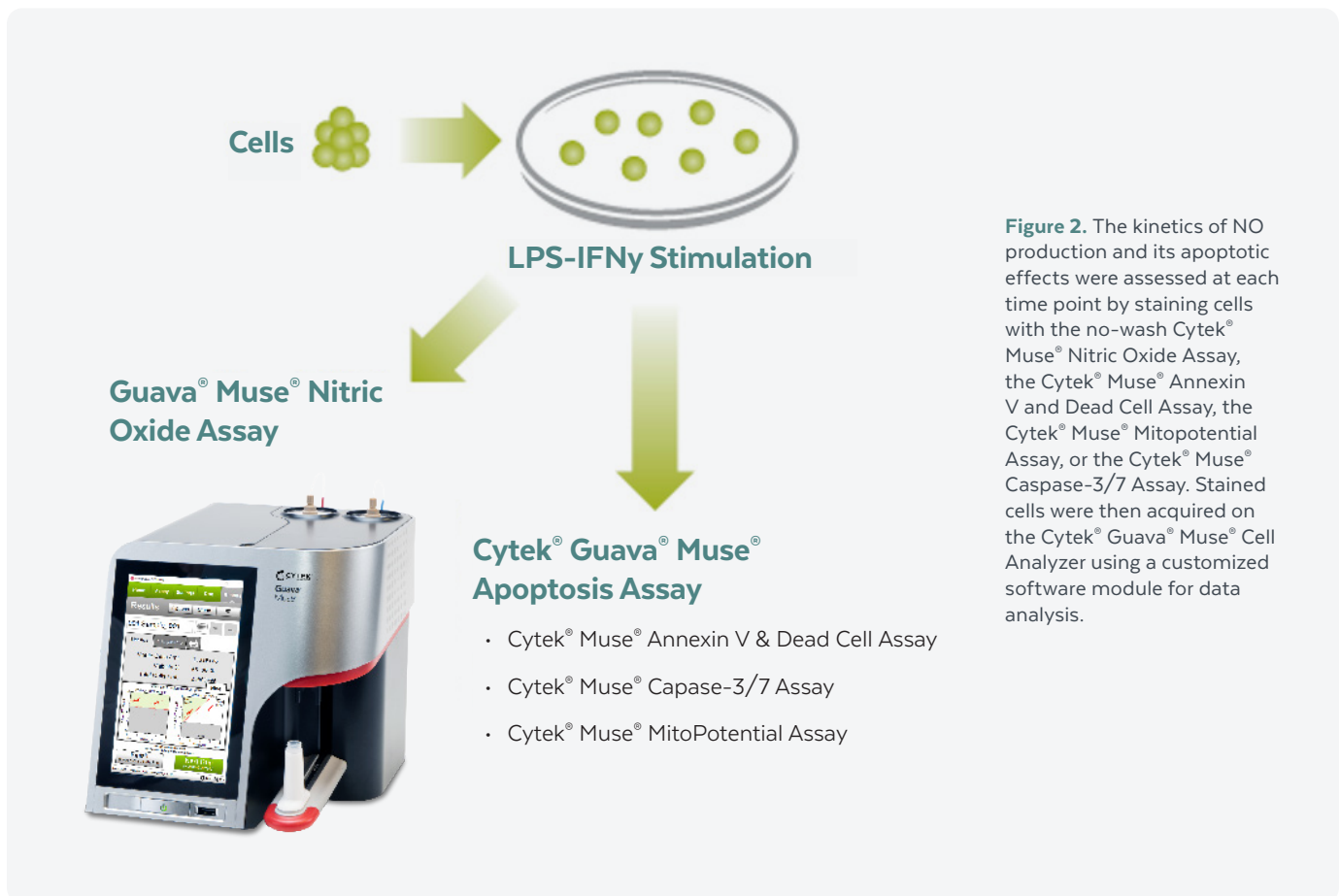
Methods

The Muse Nitric Oxide Assay measures intracellular NO levels and cell viability simultaneously. NO activity levels are quantitatively measured on an individual cell basis using the novel, membrane-permeable reagent DAX-J2™ Orange, which generates a fluorescent product upon NO oxidation in the cytoplasm. Live cells are distinguished from dead cells using 7-aminoactinomycin D (7-AAD), which fluoresces on intercalation between the base pairs of DNA in dead cells, but is excluded from live cells, which have an intact plasma membrane.

RAW264.7 macrophages were treated with 100 ng/mL LPS plus 100 U/mL mouse IFN γ for 1, 2, 4, 6, 17, 24, or 48 hours. The kinetics of NO production and its apoptotic effects were assessed at each time point by staining cells with the no-wash Muse® Nitric Oxide Assay (MCH100112), the Muse Annexin V & Dead Cell Assay (MCH100105), the Muse Mitopotential Assay (MCH100110), or the Muse Caspase-3/7 Assay (MCH100108). Stained cells were then acquired on the Muse® Cell Analyzer using assay-specific software modules (**Figure 2**).

For all assays, 1×10^6 to 1×10^7 cells/mL of treated and untreated cells were resuspended in 1X assay buffer for incubation with the appropriate Muse cell health markers according to the manufacturer's protocol. Stained cells were then acquired on the Muse Cell Analyzer. The assay-optimized software module generated the results for flow cytometric analysis of the forward scatter plus reagent fluorescence, and displayed them in a scatter plot (**Figure 3A**) and in a table (**Figure 3B**).

Figure 2. Experimental Design for Analysis of RAW264.7 Macrophages Treated with 100 ng/mL LPS and 100 U/mL Mouse IFN γ for 1, 2, 4, 6, 17, 24, or 48 hours



Results

The Muse Nitric Oxide Assay and its analysis software simultaneously quantitates the fluorescence intensity from the 7-AAD and DAX-J2 signals for each cell acquired, returning a scatter plot that can be gated into 4 subpopulations: live cells/NO-negative, live cells/NO-positive, dead cells/NO-positive, and dead cells/NO-negative.

Time course data revealed that NO-positive cells were first observed at 4 hours post-treatment (**Figure 4A** and **4B**), with a dramatic increase in the percentage of positive cells observed between 6 and 17 hours. The percentage of NO-positive cells showed a decline from 24 to 48 hours, suggesting that the duration of treatment may have impacted cell membrane integrity with the consequent loss of intracellular NO.

Figure 3. Software Output for the Nitric Oxide Assay

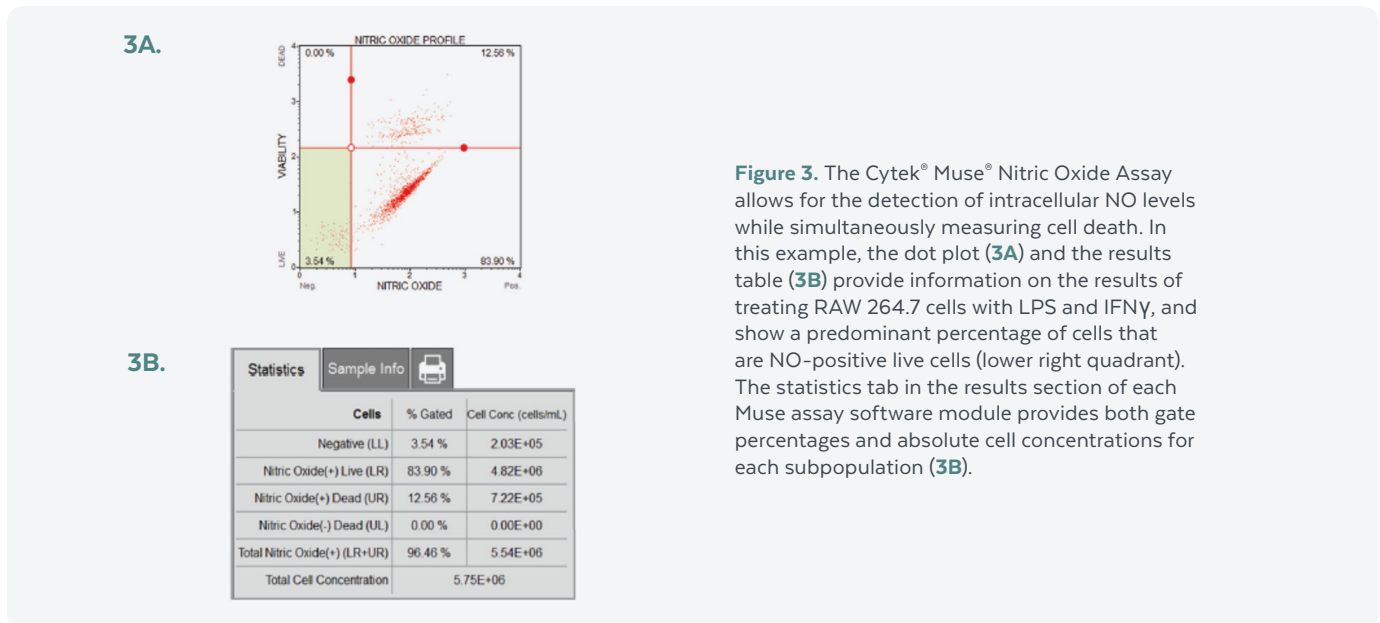
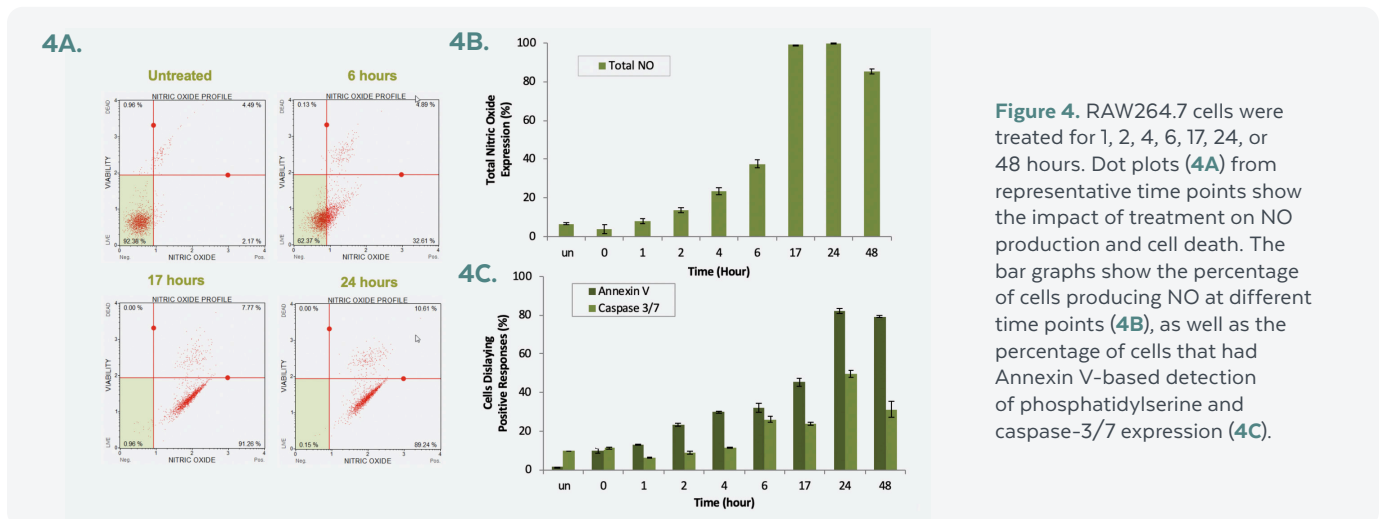


Figure 4. Time Course for NO Production and Apoptosis in LPS- and IFN γ -Stimulated RAW264.7 Macrophages Using the Cytex[®] Muse[®] Nitric Oxide Assay, the Cytex[®] Muse[®] Annexin V and Dead Cell Assay, and the Cytex[®] Muse[®] Caspase-3/7 Assay



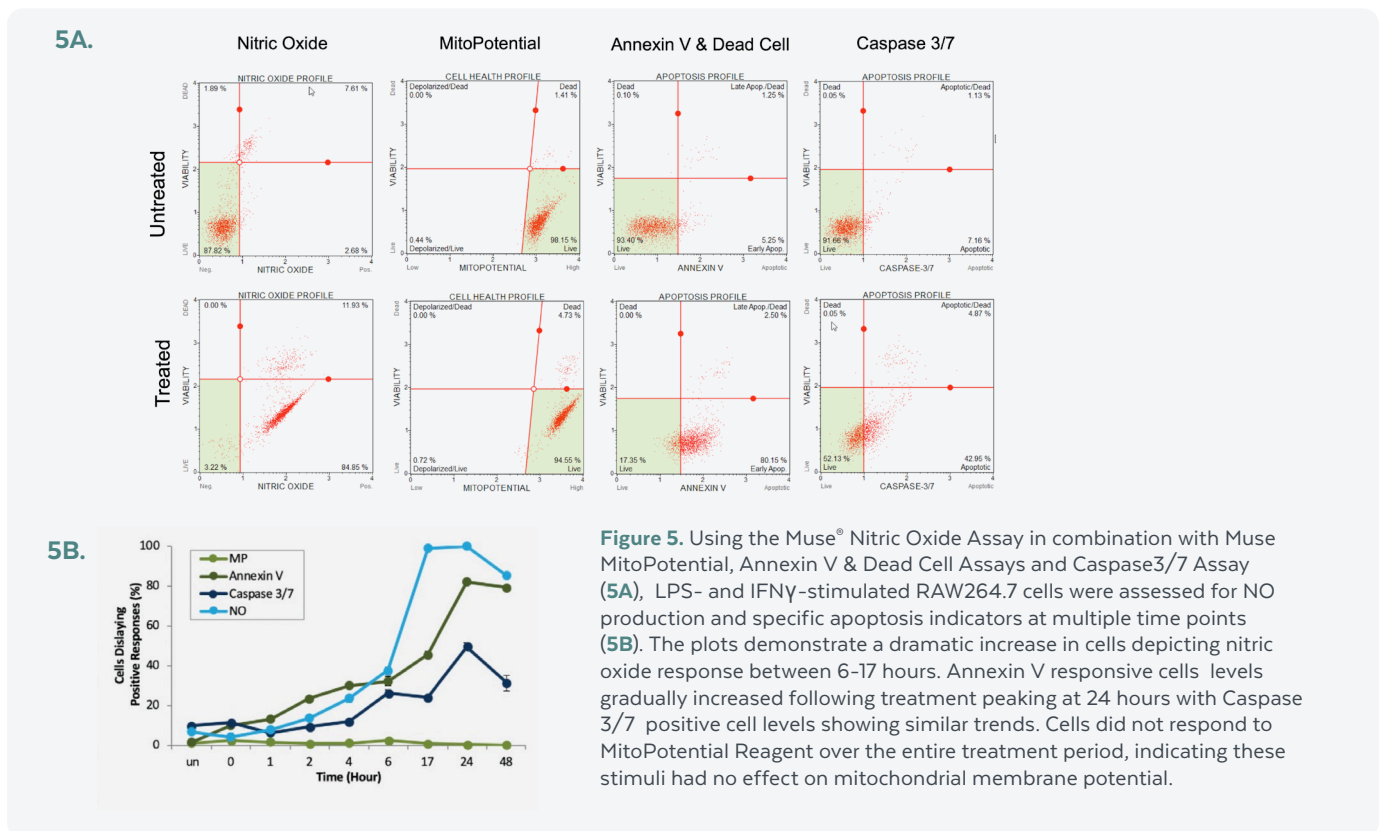
Results

LPS / IFN γ activation increases the Annexin V signal and caspase activity, but not mitochondrial depolarization.

Following stimulation with LPS and IFN γ , RAW264.7 macrophages were assessed for markers of apoptosis at 1, 2, 4, 6, 17, 24, or 48 hours. Muse assays were used for the detection of Annexin V, caspase-3/7, and mitochondrial depolarization. An increase of caspase-3/7 was first observed at 6 hours post-stimulation, with the maximum percentage of caspase-positive cells observed at 24 hours. By 48 hours, caspase-3/7 levels decreased to about 30% (Figure 4C). In contrast, surface phosphatidylserine expression—as measured by the Muse Annexin V and Dead Cell Assay—demonstrated that nearly 25% of cells were positive for this early-apoptosis marker just 2 hours after stimulation, and Annexin V levels gradually increased to over 80%, peaking at 24 hours. Caspase-3/7 expression confirmed apoptosis but demonstrated slower kinetics, with detectable expression appearing 6 hours after treatment, peaking at 24 hours when approximately 50% of the population was positive, and then decreasing significantly to approximately 30% positive when measured at 48 hours. These results demonstrate that LPS and IFN γ treatment of RAW264.7 cells not only induces NO production, but also causes significant apoptosis.

In notable contrast with other apoptosis indicators, no change in signal from the MitoPotential Assay was observed at any of the time points between 1 and 48 hours post-stimulation (Figure 5), indicating that mitochondrial membrane depolarization was not a consequence of inflammatory stimuli during this experiment. This suggests that activated macrophages may undergo apoptosis via mechanisms that do not involve intrinsic or mitochondrial-mediated pathways.

Figure 5. Multiple Assays Provide a Comprehensive Understanding of the Macrophage Response to Proinflammatory Stimuli



Conclusions

Nitric oxide generation plays a key role in several cellular processes, including cytoproliferation, host defense mechanisms, and cancer metastasis. Further, NO production may have autocrine effects on the cells that generate it, and have pro- and anti-apoptotic effects that modulate cellular responses.

By mimicking a bacterial infection, lipopolysaccharide (LPS) and interferon- γ (IFN γ) can model macrophage effector production of NO. To investigate the mechanisms that follow macrophage activation, we performed the NO detection protocol in tandem with other Muse assays that employ multiple markers to characterize cell stress and apoptotic pathways.

The results of this study demonstrate rapid kinetics for NO production, which occur in parallel with phosphatidylserine translocation and caspase-3/7 activation. Interestingly, no mitochondrial membrane depolarization ($\Delta\Psi_m$) was observed at any time during the 48-hour treatment period. This suggests that activated macrophages may proceed through nitric oxide production and cell death pathways that do not appear to involve mitochondrial depolarization.

References

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