Application Note

Kinetics of intracellular nitric oxide production and cytotoxicity in lipopolysaccharide- and interferon-γ-induced macrophage activation

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Introduction

Nitric Oxide (NO) is a small, gaseous, reactive molecule generated in a wide variety of cells that notably includes 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, its importance has been demonstrated in key processes such as cellular proliferation, apoptosis, and cancer progression, where it has been implicated in tumorigenesis, tumor killing, and metastasis in a concentration-dependent manner.

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

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

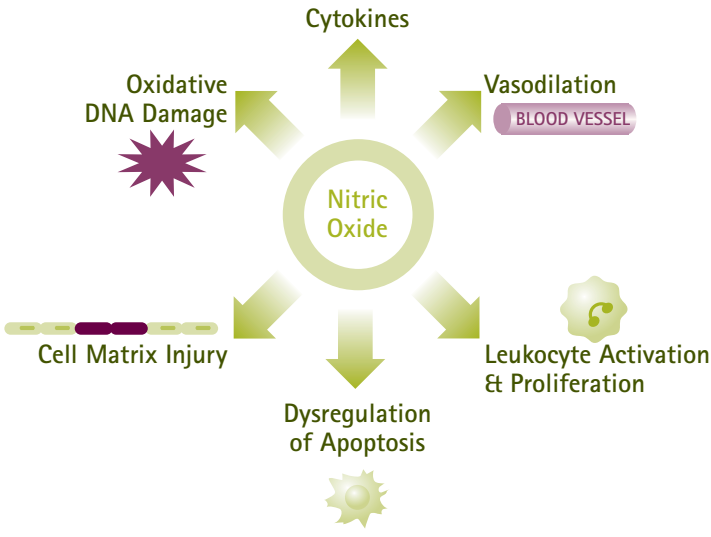

Figure 1.

Cellular functions and consequences of nitric oxide (NO) production. NO, a key effector molecule for many cell types, is generated by nitric oxide synthase (NOS)-mediated conversion of L-arginine to L-citrulline. NO modulates a remarkable variety of biochemical reactions involved in processes that include vasodilation, neurotransmission, and the immune response.

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Methods

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), Muse® Annexin V and Dead Cell kit (MCH100105), Muse® MitoPotential Assay (MCH100110), or Muse® Caspase 3/7 Kit (MCH100108), and acquired on the Muse® Cell Analyzer using assay-specific software modules (Figure 2).

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

For all assays, both untreated and treated cells were suspended in 1X assay buffer at 1 x 10^6 to 1 x 10^7 cells/mL for incubation with the appropriate Muse® fluoresces according to the manufacturer's protocol. Cells were incubated at the recommended temperature for the indicated time (≤30 minutes for all assays) before acquisition on the Muse® Cell Analyzer. Results of flow cytometric analysis of forward scatter plus reagent fluorescence are displayed by scatter plot (Figure 3A) and as summary data automatically entabulated by the software module optimized for each assay (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. The kinetics of NO production and its apoptotic effects were assessed at each time point by staining the cells with the no-wash Muse® Nitric Oxide Kit, Muse® Annexin V & Dead Cell Assay, Muse® Mitopotential Assay, and Muse® Caspase-3/7 Assay, followed by acquisition on the Muse® Cell Analyzer with a customized software module for analysis of data returned from each assay.

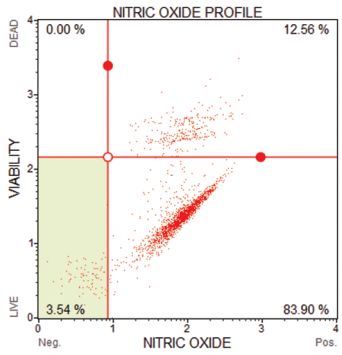
Results

Percentage of NO-positive macrophages increases rapidly from 4 to 24 hours post-stimulation

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

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

3A.



3B.

Statistics | Sample Info

Cells	% Gated	Cell Conc (cells/mL)
Negative (LL)	3.54 %	2.03E+05
Nitric Oxide(+) Live (LR)	83.90 %	4.82E+06
Nitric Oxide(+) Dead (UR)	12.56 %	7.22E+05
Nitric Oxide(-) Dead (UL)	0.00 %	0.00E+00
Total Nitric Oxide(+) (LR+UR)	96.46 %	5.54E+06
Total Cell Concentration	5.75E+06	

Figure 3.

The Muse® Nitric Oxide Assay optimizes the detection of intracellular NO levels while simultaneously measuring cell death. In this example, the dot plot and results table provide information on results of treatment of RAW 264.7 cells with LPS/IFNγ and show that a predominant percentage of cells are live cells positive for NO (lower right quadrant). The statistics tab in the results section of each Muse® assay software module entabulates both gate percentages and absolute cell concentrations for each subpopulation (3B).

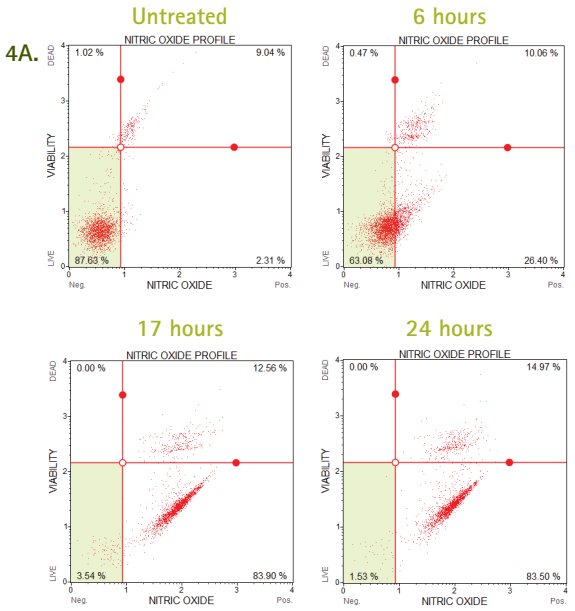


Figure 4.

Time course for NO production and apoptosis in LPS and IFN-γ stimulated RAW264.7 macrophages using the Muse® Nitric Oxide Assay, Muse® Annexin V and Dead Cell and Muse® Caspase-3/7 Assay. The data show results from the treatment of RAW264.7 cells treated for 1, 2, 4, 6, 17, 24 or 48 hours. Dot plots (4A) from representative time points show impact of treatment on nitric oxide production and cell death. Bar graphs in (4B) and (4C) show percentage of cells with NO production at different time points (4B) and percentage of cells that show Annexin V-based detection of phosphatidylserine and caspase-3/7 expression.

LPS/IFN-γ activation increases 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 the same time points described for the NO time course, using Muse® assays for the detection of Annexin V, caspase 3/7, and mitochondrial depolarization. An increase over the untreated control in caspase-3/7 was first observed at 6 hours post-stimulation, with the maximum percentage of caspase-positive cells attained at 24 hours, decreasing to about 30% caspase-positive at 48 hours (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, with a gradual increase to over 80% of cells positive for Annexin V (AV) at 24 hours. Caspase 3/7 expression confirms apoptosis but followed slower kinetics, with detectable expression observed 6 hours after treatment, peaking at 24 hours when approximately 50% the population was positive, and decreasing significantly to approximately 30% positive when measured at 48 hours. These results demonstrate that LPS/IFNy treatment of RAW 264.7 cells not only induces nitric oxide 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 at which cells were collected from 1 to 48 hours post-stimulus (Figure 5), indicating that mitochondrial membrane depolarization was not a consequence of inflammatory stimulus during this interval. This suggests that activated macrophages may undergo apoptosis via events that do not involve the intrinsic or mitochondria-mediated pathway.

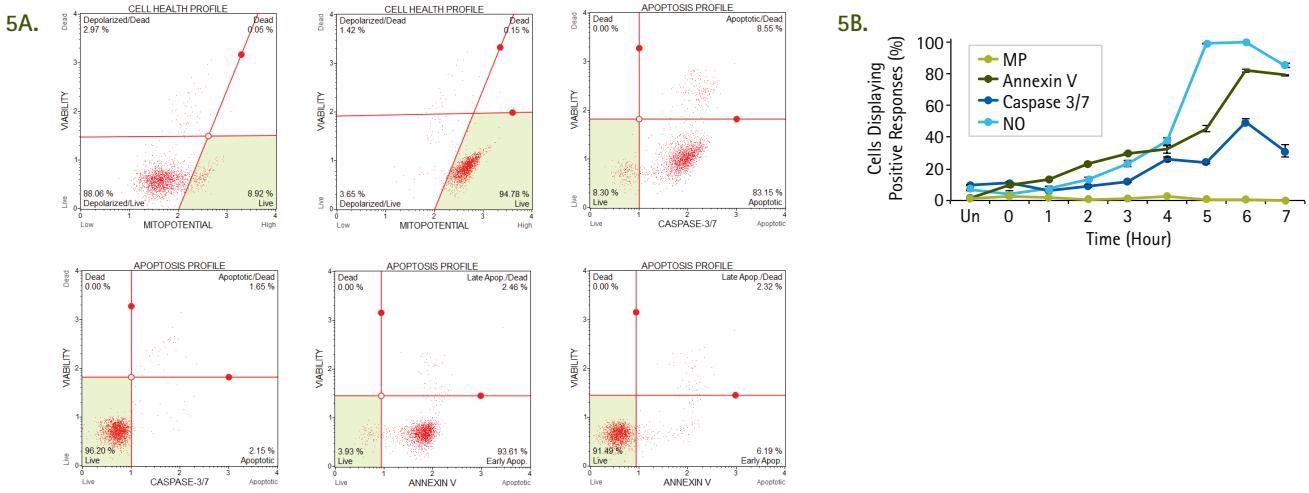


Figure 5.

Multiple assays provide a comprehensive understanding of macrophage response to proinflammatory stimuli. Using the Muse® Nitric Oxide kit in combination with the Muse® Mitopotential, Caspase 3/7 and Annexin V and Dead Cell assays (5A), macrophages activated with LPS/IFNY were assessed simultaneously for NO production and specific apoptosis indicators at multiple time points. (5B). A moderate increase in activation of caspases 3 and 7 was observed 6 hours post-stimulation, while Annexin V signal increased gradually following treatment, peaking at 24 hours after stimulus, potentially due to autocrine effects of NO production which increased dramatically between 6 and 17 hours after treatment. No Mitopotential signal increase was observed over the entire treatment period, indicating no effect of treatment on mitochondrial membrane potential.

Conclusions

Nitric oxide generation plays a key role in several cellular processes such as cytoproliferation, host defense mechanisms, and cancer metastasis. It is important to note that NO itself may have autocrine effects on the cells that produce it by exerting both pro- and anti-apoptotic effects that modulate cellular responses.

By mimicking bacterial infection, lipopolysaccharide (LPS) and interferon gamma (IFN-γ) provide a means to model macrophage effector production of nitric oxide. To investigate the sequence of events and mechanisms than ensue following macrophage activation, we performed the NO detection protocol in tandem with other Muse® assays that employ the use of multiple markers used to characterize the nature of cell stress and apoptosis 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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