In silico Kinetic Model of iNOS Expression in Macrophages

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***Abstract* - Macrophages are a key component in the host innate response and are major contributors to the proinflammatory response against pathogens. One of the key players in the proinflammatory response is induced nitric oxide synthase (iNOS), an enzyme that provides the nitric oxide needed by phagocytic cells to create reactive nitrogen species, which are highly damaging to intracellular pathogens. To model the macrophage intracellular mechanism of iNOS gene expression, we use a systems biology approach to capture the dynamics of the iNOS gene expression system stimulated by bacterial lipopolysaccharide (LPS) and IFN-γ. Our simulation results agree with *in vitro* assays of iNOS gene expression and provide a platform for further investigating the potential impact of LPS and IFN-γ variations on macrophage effector function.**

I. INTRODUCTION

Intracellular kinetic models provide an efficient and cost effective method to understand the temporal behavior of various gene expression systems in response to cell signaling molecules [1]. The macrophage proinflammatory response and consequential gene expression cascade, is a system that can be investigated using kinetic intracellular models. Schroder *et al* and MacMicking *et al* describe how the induced nitric oxide synthase (iNOS) enzyme plays a critical role in the primary proinflammatory response in macrophages upon pathogenic infection [2, 3]. Therefore, modeling the iNOS enzyme expression system can provide additional insight regarding the relationship between pathogen exposure and initiation of the macrophage effector response.

Macrophages are phagocytic cells that are able to recognize a wide array of signaling molecules such as lipopolysaccharide (LPS) from pathogens, and cytokines such as interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α). The binding of these signaling factors activates various intracellular pathways leading to the expression of proinflammatory genes that can further induce chemokine/cytokine expression or production of reactive oxygen species that directly antagonize intracellular pathogens. The latter response is carried out by either NADPH oxidase that generates superoxide anions or by the synergistic effect of iNOS that, together with NADPH oxidase, generates reactive nitrogen species, ultimately having a more profound effect on intracellular pathogens [4, 5].

In the case of iNOS expression, LPS, together with IFN-γ are the key signaling molecules that induce iNOS expression within macrophages through a synergistic mechanism. Utilizing the TLR4 and CD14 membrane receptors, LPS activates the MAP kinase pathway that proceeds to activate NF-kB, a prominent transcription factor that is normally repressed in the cytosol by the protein, IkB. MAP kinase pathway impacts various cellular regulatory mechanisms and is comprised of three sub-pathways, namely, extra-cellular receptor kinase (ERK), p38, and Jun N-terminal kinase (JNK). Recently, it has been shown that LPS activation within macrophages activates the JNK-MAPK sub-pathway ultimately leading to the expression of activator protein 1 (AP1) and NF-kB: two of the four transcription factors required for iNOS gene transcription [2, 6, 7].

IFN-γ similarly contributes to iNOS gene expression but through an alternative pathway. The cytokine activates the Janus-kinase pathway leading to the cytosolic STAT1 protein phosphorylation and dimerization (JakStat pathway). The phosphorylated STAT1 dimers translocate to the nucleus and act as transcription factors for both the iNOS gene and the interferon regulatory factor-1 (IRF-1), another key transcription factor for the iNOS gene. Ultimately, the iNOS gene expression system requires a combination of the four transcription factors expressed by the MAPK and the JakStat pathways [3, 8].

To model the complexity of the macrophage intracellular response to bacterial LPS and IFN-γ and their effect on iNOS gene expression, we developed a kinetic model that includes a comprehensive system of intermediate signaling molecules, forward and reverse reactions, and negative feedback inhibitory proteins that modulate the production of iNOS. Although there exists a previously published individual kinetic models of both the JakStat and MAPK pathways, we have formulated a model that takes into account the synergistic role of both pathways in the induction of iNOS [8, 9]. Moreover, the results obtained from our model are compared against experimental studies of iNOS gene expression in male Lewis rats treated with LPS [10].

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Our model, therefore, has the potential to predict the effects various levels of activating molecules have on the dynamics iNOS gene expression, which can provide insight regarding potential targets for immunomodulatory therapies.

II. METHODS

To mathematically model the iNOS gene expression system, we developed a system of ordinary differential equations composed of kinetic rate equations, which were solved numerically using the MatLab ODE15s solver. Parameters for the model such as initial values and kinetic rate constants were obtained from both kinetic databases (i.e. BRENDA) and published experimental results [11-13]. Furthermore, a few assumptions were made to capture the *in vivo* intracellular mechanism such as defining the kinetic platform relative to each enzymatic or signal transduction step (using Michaelis Menton kinetics vs. mass action) or in calculating the nuclear to cytoplasmic volume ratio needed for compartmentalization within the intracellular model.

To qualitatively understand which kinetic platform would work best, we first divided the model into three individual functions that captured the LPS, IFN-γ, and gene expression mechanisms separately. Yamada et al previously modeled IFN-γ stimulation of the JakStat pathway and therefore, our model included the same parameter values and equations as the Yamada model and our results were consistent. LPS however was modeled in two stages: (1) membrane activation and (2) MAPK signal transduction to IkB degradation. Membrane activation was modeled using a rapid equilibrium assumption because the concentration of LPS greatly exceeded that of the LPS binding protein (LBP). After solving for the reaction rate that included the binding of LPS to TLR4 receptor with or without CD14, the following equation was derived:

$$v = \frac{vmax_1[\alpha][\beta][\gamma]+vmax_2 K_\alpha K_\beta[\alpha][\gamma]}{K_\alpha K_\beta K_\gamma + K_\beta K_\gamma[\alpha]+K_\gamma[\alpha][\beta]+[\alpha][\beta][\gamma]} \quad (1)$$

α = LPS; **β** = CD14; **γ** = TLR4-MD2 membrane complex
$\mathbf{K_\alpha}$ = dissociation constant of LPS to LBP:
$\mathbf{K_\beta}$ = dissociation constant of CD14 to LPS_LBP
$\mathbf{K_\gamma}$ = dissociation constant of CD14_LPS_LBP binding to TLR4-MD2

We assumed LBP as the activating protein to derive equation (1). In addition, the first term in the numerator of equation 1 highlights the additive effect of CD14 with a higher maximum velocity rate of binding to the TLR4-MD2 receptor once bound to LBP-LPS.

Although the remaining MAPK signal transduction pathway was composed of kinases and protein phosphatases, Michaelis Menton kinetics or rapid equilibrium kinetic assumptions were not used as the substrate concentration did not significantly exceed the enzyme concentration. Consequently we used mass action kinetics to formulate the remaining pathway model.

Gene expression was modeled similar to the activated membrane receptor using the rapid equilibrium assumption. Of the four transcriptions factors expressed, only IRF1, NF-kB, and AP1 are required for the initial activation of transcription whereas the phosphorylated STAT1 dimers provide an additive effect. Furthermore, the iNOS gene promoter contains two AP1 sites and two NF-kB sites. Therefore, Equation 2 captures the full dynamics of iNOS gene expression, incorporating the compounding effect in the second AP1 and NF-kB term.

$$v = \frac{vmax_1 K_1[w][x][y]+vmax_2[w]^2[x]^2[y][z]}{K_1K_2+K_2[w][x][y]+[w]^2[x]^2[y][z]} \quad (2)$$

w = NF-kB; **x** = AP1; y = IRF1; **z** = STAT1dimer-P
$\mathbf{K_1}$ = cumulative dissociation constant of NF-kB, AP1, and IRF1
$\mathbf{K_2}$ = cumulative dissociation constant of all four factors

III. RESULTS

We implemented our iNOS gene expression model in MatLab and solved using the ODE15s solver. The model was simulated for 14000 seconds to capture the steady state. We then compared our simulated results of iNOS nuclear mRNA expression to experimental results and as Figure 1 shows, the behavior of our model is comparable to empirical observations [10]. Although the experimental graph was measured in units of femtomoles/gram and our model in nano-Molar, the actual values when converted to similar units prove to be quite similar (data not shown).

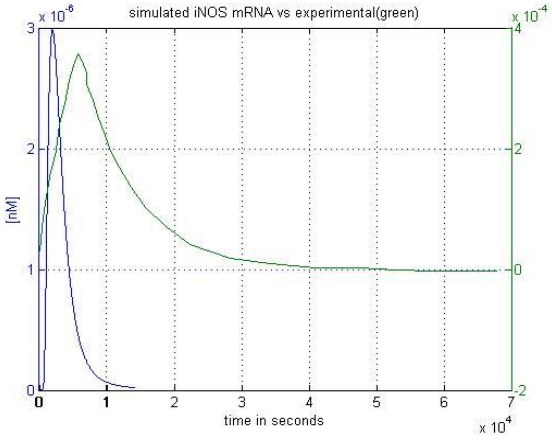

Figure 1: iNOS mRNA expression. Simulated iNOS mRNA expression (Blue), modeled using MatLab using parameters extracted from literature and kinetic databases, is plotted against experimental iNOS mRNA expression (green). Simulated expression results are inclusive of LPS and IFN-γ stimulation whereas experimental is inclusive of only LPS stimulation.

Although the time to reach maximum mRNA expression in the experimental results takes approximately one and half hours longer, only LPS stimulation is used in the *in vitro* model. Our model represents stimulation by both IFN-γ and LPS, which may contribute to the gene expression in our model reaching a maximum value faster as a result of higher cumulative activator concentration. The steep decline in iNOS mRNA production is likely a result of IFN-γ and LPS depletion. The observed behavior of our model appears consistent with *in vivo* macrophage inflammatory response, namely the increased production of effector molecules in the presence of cytokines produced by multiple innate and adaptive immune cells such as natural killer cells and T-cells respectively [14].

IV. CONCLUSION

We developed a kinetic model to simulate iNOS gene expression patterns in murine macrophages. Although our results displayed similar behavior as *in vitro* results, our model exhibited a faster response time and reached maximum gene expression earlier than the *in vitro* system. Future work will leverage empirical data from *in vitro* studies conducted by our lab to obtain optimized parameter values that will correlate to an *in vitro* and ultimately *in vivo* response mechanism for the model.

Our current model provides a theoretical *in silico* platform to explore the effect of LPS and IFN-γ on iNOS gene expression. As an example, initial studies indicate that variations in the concentrations of either LPS or IFN-γ showed positive correlation with simulated gene expression patterns. In addition, our model can be expanded to explore the impact of gene deletions or inhibitory molecules on the dynamics of iNOS expression. Results from these simulations can provide insight for targeted drug therapies as well as insight into potentially adverse side of effects of immunosuppressive therapy.

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