Identification of a Novel Pathway of Transforming Growth Factor-β1 Regulation by Extracellular NAD⁺ in Mouse Macrophages

IN VITRO AND IN SILICO STUDIES

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Background: Both extracellular NAD⁺ and the cytokine TGF-β1 are anti-inflammatory.

Results: NAD⁺ increases both active and latent TGF-β1 in mouse macrophages. A mathematical model partially explains the complex effects of NAD⁺ on TGF-β1.

Conclusion: NAD⁺ is a novel modulator of TGF-β1.

Significance: Combined in vitro and in silico approaches may help elucidate novel pathways of TGF-β1 regulation.

Extracellular β-nicotinamide adenine dinucleotide (NAD⁺) is anti-inflammatory. We hypothesized that NAD⁺ would modulate the anti-inflammatory cytokine Transforming Growth Factor (TGF)-β1. Indeed, NAD⁺ led to increases in both active and latent cell-associated TGF-β1 in RAW 264.7 mouse macrophages as well as in primary peritoneal macrophages isolated from both C3H/HeJ (TLR4-mutant) and C3H/HeOuJ (wild-type controls for C3H/HeJ) mice. NAD⁺ acts partially via cyclic ADP-ribose (cADPR) and subsequent release of Ca²⁺. Treatment of macrophages with the cADPR analog 3-deaza-cADPR or Ca²⁺ ionophores recapitulated the effects of NAD⁺ on TGF-β1, whereas the cADPR antagonist 8-Br-cADPR, Ca²⁺ chelation, and antagonism of L-type Ca²⁺ channels suppressed these effects. The time and dose effects of NAD⁺ on TGF-β1 were complex and could be modeled both statistically and mathematically. Model-predicted levels of TGF-β1 protein and mRNA were largely confirmed experimentally but also suggested the presence of other mechanisms of regulation of TGF-β1 by NAD⁺. Thus, in vitro and in silico evidence points to NAD⁺ as a novel modulator of TGF-β1.

Inflammation is a complex process in which various potent mechanisms that can control infection, injury, and proliferative diseases must be kept in check (1). Studies over the past decade have focused on the release of damage-associated molecular patterns (DAMPs) from cells, a class of molecules that signal a disruption of cellular homeostasis. Prototypically, these DAMPs are proteins or other cellular constituents that carry out housekeeping functions normally but are released in settings of stress, inflammation, or injury. In turn, these agents stimulate, propagate, or potentiate both innate and adaptive immune responses (2). Recent studies have suggested that one such mediator may be β-nicotinamide adenine dinucleotide (NAD⁺), a ubiquitous cellular constituent that is used by cells as an electron acceptor (or, in its reduced form, NADH, as an electron donor) in a wide variety of enzyme-catalyzed redox reactions. These actions of NAD⁺ occur in multiple cell types secondary to the formation of cyclic adenosine dinucleotide ribose (cADPR) from NAD⁺, with subsequent release of Ca²⁺ (3). Importantly, NAD⁺ has been found to exert a profound anti-inflammatory activity that it appears to share with nicotinamide (4). The mechanisms by which these anti-inflammatory actions are carried out, however, remain poorly understood.

Transforming Growth Factor β1 (TGF-β1) is a cytokine that belongs to a family of three related isoforms, all of which exert crucial biological functions. Of these three isoforms, TGF-β1 is the most prominent in the control of inflammation and immunity (5). The numerous biological functions of all TGF-βs require a set of post-translational modifications termed “activation.” The bioactive forms of all TGF-βs are 25-kDa homodimers produced from ~50-kDa monomers that dimerize to form the ~100-kDa TGF-β precursor. This precursor is cleaved intracellularly by furin proteases to yield the 25-kDa active TGF-β dimer, which remains associated with the

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4 The abbreviations used are: cyclic DAMP, damage-associated molecular pattern; 3-cADPR, 3-deaza-adenosine diphosphate ribose; BAPTA-AM, 1,2-bis-(o-aminophenoxy)-ethane-N,N',N''-tetraacetic acid tetraacetoxy-methyl ester; ODE, ordinary differential equation.
Effects of NAD\(^+\) on Macrophage TGF-β1

remaining portion of its own pro-form, the latency-associated peptide (∼75 kDa). This complex is termed "latent TGF-β" and is secreted in this form. Other proteins, such as latent TGF-β-binding proteins (LTBP, which targets TGF-β to the extracellular matrix) or α2 macroglobulin (which is associated with circulating TGF-β1) can bind to this complex, creating the so-called large latent complex. Latent TGF-β is activated by a process that involves dissociation and degradation of latency-associated peptide by proteins (e.g. plasmin and transglutaminase), heat, chaotropic agents, and acid as well as oxygen and nitrogen free radicals (6, 7). Although TGF-β1 can autoinduce its own expression at the mRNA level, the post-translational control of TGF-β1 through activation is arguably the most potent regulatory mechanism for this cytokine (6, 7).

As early as 1978, an "NAD\(^+\)-splitting enzyme" was reported in macrophages (8–10). Because cytokines, radiation, and free radicals can lead to the activation and increased expression of latent TGF-β1 in macrophages (11–14), we hypothesized that extracellular NAD\(^+\) could exert a similar effect. We further hypothesized that the mechanism by which NAD\(^+\) would act would involve the generation of cADPR from extracellular NAD\(^+\). Finally, we hypothesized that cADPR would exert its effects via the stimulation of Ca\(^{2+}\). To better understand the complex interplay among NAD\(^+\), cADPR, Ca\(^{2+}\), and active and latent TGF-β1, we constructed both statistical and mathematical models and validated some predictions from these models in vitro. These computational models also suggested the existence of as yet unknown mechanisms by which NAD\(^+\) can augment TGF-β1. Taken together, our results demonstrate a novel pathway for TGF-β1 activation via NAD\(^+\) and its metabolites and highlight the utility of mathematical modeling for discovering novel biological mechanisms.

**EXPERIMENTAL PROCEDURES**

**Reagents**—The DMEM and HEPES buffer used for cell culture were purchased from BioWhittaker-Lonza (Baltimore, MD). The penicillin/streptomycin and the L-glutamine media additives were purchased from Invitrogen. Fetal bovine serum was purchased from Gemini BioProducts (West Sacramento, CA) and was used due to its low TGF-β1 content (data not shown). Antibodies to human TGF-β1 and human latency-associated peptide were purchased from R&D Systems (Minneapolis, MN). The secondary antibodies, the normal serum, the ABC enzyme conjugate kit, and the diaminobenzidine brown chromogen peroxide solution for 30 min. Nonspecific antibody reactivity was blocked as follows. The wells that were stained for active TGF-β1 were blocked with 1.5% goat serum, whereas the wells stained for latent TGF-β1 were blocked with 1.5% rabbit serum; all blocking was performed for 20 min. The cells were then incubated with primary antibody specific for either active TGF-β1 (chicken anti-human active TGF-β1) or latent TGF-β1 (goat anti-human latent TGF-β1) for 30 min. The cells were then incubated with secondary antibodies as follows. For wells stained for active TGF-β1, the secondary antibody solution consisted of ∼0.5% goat anti-chicken antibody mixed with 1.5% goat serum and 1 ml of PBS. For wells stained for latent TGF-β1, the secondary antibody solution consisted of 0.5% rabbit anti-goat antibody mixed with 1.5% rabbit serum and 1 ml of PBS. The incubation time for the secondary antibody was 30 min. The cells were then exposed to the ABC enzyme con-
Effects of NAD$^+$ on Macrophage TGF-β1

In Primary Mouse Peritoneal Macrophages—We next sought to confirm in primary macrophages the basic finding of NAD$^+$/cADPR-mediated action of latent TGF-β1. NAD$^+$ increases the expression of active and latent TGF-β1 in RAW 264.7 mouse macrophage-like cell line. Various studies have suggested that the activation of latent TGF-β1 is often best assessed immuno- cytochemically (13–15), and so we utilized this method initially. In prior studies (15), we have shown concordance between immunoblotting and this immunocytochemistry-based method of differential detection of active versus latent TGF-β1. As shown in Fig. 1, treatment with 10–1000 μM NAD$^+$ for 2 h led to a dose-dependent increase in immunocytochemically detectable latent (panels B–D) and active (panels F–H) TGF-β1 compared with control resting cells (panels A and E).

NAD$^+$ and Its Extracellular Metabolite cADPR Increase the Expression of Active and Latent TGF-β1 in Primary Mouse Peritoneal Macrophages—We next sought to confirm in primary macrophages the basic finding of NAD$^+$/cADPR-mediated action of latent TGF-β1. NAD$^+$ increases the expression of active and latent TGF-β1 in RAW 264.7 mouse macrophage-like cell line. Various studies have suggested that the activation of latent TGF-β1 is often best assessed immuno- cytochemically (13–15), and so we utilized this method initially. In prior studies (15), we have shown concordance between immunoblotting and this immunocytochemistry-based method of differential detection of active versus latent TGF-β1. As shown in Fig. 1, treatment with 10–1000 μM NAD$^+$ for 2 h led to a dose-dependent increase in immunocytochemically detectable latent (panels B–D) and active (panels F–H) TGF-β1 compared with control resting cells (panels A and E).

RESULTS

NAD$^+$ Increases the Expression of Active and Latent TGF-β1 in RAW 264.7 Macrophage-like Cells—We first tested the hypothesis that treatment of macrophages with extracellular NAD$^+$ would lead to increased expression of both active and latent TGF-β1 in macropores, utilizing the RAW 264.7 mouse macrophage-like cell line. Various studies have suggested that the activation of latent TGF-β1 is often best assessed immuno- cytochemically (13–15), and so we utilized this method initially. In prior studies (15), we have shown concordance between immunoblotting and this immunocytochemistry-based method of differential detection of active versus latent TGF-β1. As shown in Fig. 1, treatment with 10–1000 μM NAD$^+$ for 2 h led to a dose-dependent increase in immunocytochemically detectable latent (panels B–D) and active (panels F–H) TGF-β1 compared with control resting cells (panels A and E).

NAD$^+$ and Its Extracellular Metabolite cADPR Increase the Expression of Active and Latent TGF-β1 in Primary Mouse Peritoneal Macrophages—We next sought to confirm in primary macrophages the basic finding of NAD$^+$/cADPR-mediated action of latent TGF-β1. NAD$^+$ increases the expression of active and latent TGF-β1 in RAW 264.7 mouse macrophage-like cell line. Various studies have suggested that the activation of latent TGF-β1 is often best assessed immuno- cytochemically (13–15), and so we utilized this method initially. In prior studies (15), we have shown concordance between immunoblotting and this immunocytochemistry-based method of differential detection of active versus latent TGF-β1. As shown in Fig. 1, treatment with 10–1000 μM NAD$^+$ for 2 h led to a dose-dependent increase in immunocytochemically detectable latent (panels B–D) and active (panels F–H) TGF-β1 compared with control resting cells (panels A and E).
Effects of NAD⁺ on Macrophage TGF-β1

FIGURE 1. Increased expression of active and latent TGF-β1 protein induced by NAD⁺ in RAW 264.7 macrophage-like cells. Mouse RAW 264.7 cells were either incubated with medium alone (panels A and E) or were treated with 10, 100, or 1000 µM NAD⁺ for 2 h (panels B–D and F–H) as indicated and subsequently immunostained for latent (panels A–D) or active (panels E–H) TGF-β1 as described under “Experimental Procedures.”

increase in TGF-β1 and to determine whether or not the effect of NAD⁺/cADPR was due to LPS contamination, again using our previously published immunocytochemical method coupled with image analysis and quantification (15).

As seen in Fig. 2, treatment with either 100 µM NAD⁺ or 10 nm stable cADPR analog 3-deaza-cADPR (17) for 1 h led to increased active (Fig. 2, A and B) and latent TGF-β1 (Fig. 2C) in isolated peritoneal macrophages from both wild-type (C3H/HeOuJ) and LPS-hyporesponsive, TLR4-mutant (C3H/HeJ) mice at 1 h post-treatment. Moreover, there were no statistically significant differences between C3H/HeJ and C3H/HeOuJ macrophages with regard to expression of NAD⁺/cADPR-induced active TGF-β1 (Fig. 2B). Taken together, these results show that NAD⁺ and 3-cADPR can induce and activate TGF-β1 in primary macrophages in vitro and strongly suggest that LPS contamination is at most an extremely minor contributor to the effects of NAD⁺ and 3-cADPR on TGF-β1.

The Extracellular NAD⁺ Metabolite cADPR Increases the Expression of Both Active and Latent TGF-β1 in RAW 264.7 Macrophages—As seen in Fig. 3A, treatment with 100 µM NAD⁺ for 2 h led to increased active TGF-β1. To determine whether this effect of NAD⁺ depends on the prior conversion to cADPR (3), we treated RAW 264.7 cells with NAD⁺ in the presence of the stable, cell-permeable cADPR antagonist 8-Br-cADPR (16). As seen in Fig. 3A, 10 µM 8-Br-cADPR antagonized the effect of NAD⁺ on both active and latent TGF-β1.

We also observed a significant expression of active (but not latent) TGF-β1 induced by this dose of 8-Br-cADPR, which we hypothesize is due to an off-target effect of this compound.

We next sought to further define the TGF-β1 response of RAW 264.7 cells to cADPR. As seen in Fig. 3B, authentic cADPR (10 µM) led to increased expression of both active and latent TGF-β1. Similarly, the stable cADPR analog 3-deaza-cADPR (17) (which is much more stable than authentic cADPR) also led to increased expression of both active (Fig. 3C) and latent TGF-β1 (Fig. 3D) at concentrations of 1–50 nm.

The Effects of NAD⁺ and cADPR on TGF-β1 Require Ca²⁺ Mobilization, Are Mimicked by Ca²⁺ Agonists, and Are Inhibited by Ca²⁺ Antagonist—cADPR has been reported to lead to the release of Ca²⁺ from ryanodine-sensitive intracellular stores (3). Accordingly, we further probed this pathway in the response to NAD⁺. We found that blocking Ca²⁺ with the Ca²⁺ chelator BAPTA (10 µM) inhibited the activation of TGF-β1 by 100 µM NAD⁺ (Fig. 4A) at 1 h and to a lesser degree at 3 h (Fig. 4B). Furthermore, treatment with the cADPR analog 3-cADPR (1 nm, Fig. 5A) and the Ca²⁺ agonists bradykinin (10 µM, Fig. 5B) or ionomycin (1 µM, Fig. 5C) for 1 h induced increased active and latent TGF-β1, an effect inhibited by pretreatment for 30 min with the Ca²⁺ chelator BAPTA. The involvement of Ca²⁺ mobilization in the activation of TGF-β1 was further investigated using the L-type calcium channel blocker verapamil. We found that pretreatment of macrophages with 100 µM verapamil for 30 min resulted in a significant inhibition of NAD⁺-induced activation of TGF-β1 (Fig. 5D), although this drug had no effect on the immunostaining for latent TGF-β1.

The Effect of NAD⁺ on Active and Latent TGF-β1 Follows a Complex Dose and Time Course—We next carried out a detailed dose- and time-course study of the effects of NAD⁺ on the immunocytochemically detectable expression of active (Fig. 6A) and latent TGF-β1 (Fig. 6D). This study suggested that the effect of NAD⁺ on both active and latent TGF-β1 was complex and possibly biphasic. NAD⁺ led to the activation of latent TGF-β1 at early time points (1–2 h), which declined by 6 h and then appeared to rise again toward 24 h. The effect on latent (total) TGF-β1 was similar but shifted in time, with the peak effect of NAD⁺ on latent TGF-β1 occurring at 6 h and then declining by 12 h. This study also suggested that the effect of NAD⁺ on active TGF-β1 was dose-dependent from 10 to 1000 µM at late time points (8–24 h) post-stimulation but peaked at 100 µM and declined at 1000 µM at early time points (1–6 h). The effects on NAD⁺ on latent TGF-β1 were similar but again appeared to be shifted in time, with dose-dependent increases of latent TGF-β1 apparent at 1, 12, and 24 h but with a peak at 100 µM at 2–8 h.

Statistical and Mathematical Modeling of the Complex Effects of NAD⁺/cADPR/Ca²⁺ on TGF-β1—To gain insight into the complex dose- and time-courses described above, the responses were modeled statistically (see Figs. 6, B–E) as a bivariate gaussian vector (see “Experimental Procedures”). The expected value of active TGF-β1 was modeled as
E(\text{active TGF-1}) = 1.74 + 0.75 \text{ CI} - 0.5 \text{ Cq} + 0.49 \text{ Tq} \\
+ 1.07 \text{ CqTI} - 0.8 \text{ CqTq} \quad \text{(Eq. 1)}

Upper bounds on the \( p \) values of these coefficients based on \( t \) tests and read from left to right are, respectively, 0.0001, 0.0001, 0.005, 0.025, 0.015, and 0.068. The model-based estimate for the variance was \( S^2 = 1.09 \) on 142 degrees of freedom. The \( R^2 \) was 0.233. The hypothesis that there are no effects whatsoever upon this response was rejected based on an \( F \) value of 5.38 on 8 and 142 degrees of freedom with a \( p \) value of 0.00000644. From this model we concluded that active TGF-1 is significantly influenced primarily by the concentration of NAD\(^+\), which manifests both linear and quadratic influence by a quadratic effect of time and by two significant concentration-time interactions.

Dependence of latent TGF-1 on NAD\(^+\) was deemed to be simpler in the sense that interactions between concentration and time were not required in the model. Indeed, in this case we obtained a purely linear dependence. The following equation describes the relationship between latent TGF-1, NAD\(^+\) concentration, and time,

\[ E(\text{latent TGF-1}) = 1.85 + 0.97 \text{ CI} - 0.5 \text{ Cq} - 0.35 \text{ TI} \quad \text{(Eq. 2)} \]

The analogous \( p \) values, read from left to right, are lower than the respective values of 0.0001, 0.0001, 0.005, and 0.108. The model-based \( S^2 \) was 1.08 on 142 degrees of freedom with an \( R^2 \) of 0.238. The hypothesis of no effects of concentration and time upon latent TGF-1 was rejected based on an \( F \) value of 5.554 on 8 and 142 degrees of freedom with a \( p \) value of 0.00000405. This statistical model suggested that the effects of NAD\(^+\) on TGF-1 were nonlinear, and this model was capable of predicting not only the levels of active (Fig. 6, \( B \) versus \( A \)) and latent TGF-1 (Fig. 6, \( E \) versus \( D \)) but also the biphasic dose effect of NAD\(^+\).

To help define the mechanism by which NAD\(^+\), cADPR, and Ca\(^{2+}\) led to the induction and activation of TGF-1, we sought...
to create a mechanistic mathematical model of the presumptive interactions among these variables. Based on these data-driven modeling studies, we inferred that the effects of NAD\(^+\) on TGF-\(\beta\)1 are nonlinear. Accordingly, we created a nonlinear ODE model of interactions we considered the most parsimonious and yet still capable of recapitulating the complex biological phenomena described above. Our initial ODE model included the interactions depicted in supplemental Fig. S1B (see “Experimental Procedures”), which shows NAD\(^+\) signaling through cADPR and Ca\(^{2+}\) to activate TGF-\(\beta\)1, which can then autoinduce its own mRNA production. However, this initial model was unable to account for the apparent second rise in TGF-\(\beta\)1 observed at the later time points in Fig. 6, A and D. Importantly, we sought to include a mechanism by which BAPTA could suppress the NAD\(^+\)-induced increase in both active and latent TGF-\(\beta\)1 at 1 h (Fig. 4A), but to a lesser extent at 3 h (Fig. 4B), arriving at the model depicted in supplemental Fig. S1C. Simulations from this subsequent model were able to reproduce the experimentally observed time-dependent response of TGF-\(\beta\)1. This model could account for the timing-based difference in the effect of BAPTA on NAD\(^+\)-induced active and latent TGF-\(\beta\)1 but could not recapitulate the attenuated response seen at high dose at the early time points (Fig. 6, A and D). To explain this phenomenon, we hypothesized a threshold-dependent inhibitory effect of Ca\(^{2+}\) on TGF-\(\beta\)1 mRNA that was included in our final model (supplemental Fig. S1D) described by the following equations:

\[
\frac{dN}{dt} = -k_1N - k_{ADP}N \tag{Eq. 3}
\]

\[
\frac{dC}{dt} = k_1N - k_{cADPR}C + \beta_c \tag{Eq. 4}
\]

\[
\frac{dT_L}{dt} = -k_{T_L}T_L - k_{act}T_L C + k_{T_m}T_m \tag{Eq. 5}
\]

\[
\frac{dT_A}{dt} = -k_{T_A}T_A + k_{act}T_L C \tag{Eq. 6}
\]

\[
\frac{dX_1}{dt} = k \frac{N^*}{K^* + N} - k_{xADP}X_1 \tag{Eq. 7}
\]

\[
\frac{dX_2}{dt} = k_2X_1 - k_{xX_2}X_2 \tag{Eq. 8}
\]
Effects of NAD$^+$ on Macrophage TGF-β1

Parameter values for this model were estimated manually and are presented in Table 1. An initial simulation was carried out with no NAD$^+$ input, and the resulting values were used as the initial conditions for each of the subsequent simulations with varying concentrations of NAD$^+$ input. This procedure allows the system to equilibrate to a steady state before making any perturbations and corresponds to the culture of RAW 264.7 cells to allow for adherence (supplemental Fig. S2).

The ODE model simulations were generally in good concordance with experimental data for both active TGF-β1 (Fig. 6, C versus A) and latent TGF-β1 (Fig. 6, F versus D), although we observed a discrepancy at early time points (1 and 2 h) for latent TGF-β1 (Fig. 6, F versus D).

We next attempted to validate the predictions of both the statistical and ODE models experimentally at both the protein and mRNA levels. We first subjected RAW 264.7 cells to 10, 100, or 1000 μM NAD$^+$ for 16 h, a time point not used for the calibration of either model. As seen in Figs. 7, A and B, the statistical model was able to accurately predict the levels of active and latent TGF-β1. The ODE model was able to recapitulate the increase in active and latent TGF-β1 induced by 10 and 100 μM NAD$^+$, respectively, but was unable to capture the attenuated response to the 1000 μM NAD$^+$ dose observed at 16 h.

**NAD$^+$ Increases the Expression of TGF-β1 mRNA in a Manner Predicted in Silico**—We next examined the effects of NAD$^+$ on TGF-β1 mRNA. TGF-β1 can auto-induce its own mRNA expression (6), and this auto-induction is presumably driven by exposure of cells to active TGF-β1. Because extracellular NAD$^+$ led to the activation of latent TGF-β1 protein and the increased expression of latent (total) TGF-β1 protein, we hypothesized that exposure to NAD$^+$ would lead to increased TGF-β1 mRNA as well. Moreover, because the maximal activation of TGF-β1 by NAD$^+$ occurred at 1 h, whereas maximal NAD$^+$-stimulated latent TGF-β1 expression was seen at 6 h but persisted to 24 h, we examined the effects of NAD$^+$ on TGF-β1 mRNA at 21 h. As seen in Fig. 7C, increased expression of TGF-β1 mRNA was observed with as little as 10 μM NAD$^+$ and persisted at levels of NAD$^+$ up to 1000 μM. As described in supplemental Fig. S1B (see above), a mathematical model that invoked only the auto-induction of TGF-β1 was incapable of replicating the persistence of elevated TGF-β1 mRNA and protein at later time points observed experimentally. In contrast, a mathematical model that included a hypothetical, Ca$^{2+}$-independent pathway acting at the level of TGF-β1 mRNA induction (supplemental Fig. S1D) accurately predicted the experimental data on NAD$^+$-mediated TGF-β1 mRNA induction (Fig. 7C).

**DISCUSSION**

A large and growing class of DAMPs has emerged over the past decade as proinflammatory endogenous cellular products that stimulate inflammation (e.g. the production of TNF-α when released in settings of cell stress or cell death) (2). Canonical DAMPs include high mobility group box-1 (HMGB1), S100A, uric acid, and heat shock protein-70 (HSP-70) (2). However, relatively little attention has been paid to endogenous anti-inflammatory compounds derived from cells, mediators that include adenosine (18), ubiquitin (19), and hemopexin (20). Herein, we have identified and partially characterized a novel pathway of TGF-β1 regulation by extracellular NAD$^+$ in mouse macrophage-like cells using combined biochemical and in silico methods (Fig. 8), and our results suggest that NAD$^+$ may be another member of this emerging group of anti-inflammatory DAMPs.

Indeed, emerging literature raises the possibility that NAD$^+$ could be released by parenchymal cells in the setting of cellular stress or injury (3) and to exert various effects on inflammation. Granulocytes treated with NAD$^+$ exhibited increased production of reactive oxygen species and other features consistent with activation (21). There is also extensive evidence that T-cell signaling is affected by NAD$^+$, with ultimate effects on proliferation and apoptosis (22–28). Furthermore, several studies have shown that NAD$^+$ can protect against inflammatory stress...
both in vitro and in vivo (29, 30), in part via reduced activity of the central inflammatory transcription factor NF-κB. We have also found that treatment of endotoxemic mice with NAD⁺ could protect from lethality in the case of mice injected with high dose LPSs and greatly reduced plasma TNF-α and NOₓ/NO₃ and elevated IL-10 in mice subjected to low-dose LPS. Based on these data, we hypothesized that NAD⁺, like other DAMPs, would have to act on nearby macrophages to induce classical anti-inflammatory cytokines. Given the long-recognized, potent and generally localized effects of TGF-β1 in inflammatory settings (5), we hypothesized that one mechanism by which NAD⁺ could exert such potent effects would be via modulation of this cytokine.

The canonical signaling intermediary that controls the extracellular functions of NAD⁺ appears to be cADPR, which has been reported to lead to the release of Ca²⁺ from ryanodine-sensitive intracellular stores (3). In most models of extracellular signal transduction, NAD⁺ is converted to cADPR and nicotinamide by extracellular ADP-ribosyl cyclase (3). The cADPR then enters cells through either a CD38-dependent (31) or -independent (32) mechanisms. The resulting increase in intracellular cADPR concentration leads to binding of cADPR to ryanodine-sensitive calcium channels on endoplasmic reticulum membranes. This binding increases the probability that the channel will be in an open conformation, allowing the release of endoplasmic reticulum calcium stores to the cytoplasm (3). Although a role for extracellular cADPR in intracellular signaling events has been reported, there are other mechanisms through which extracellular NAD⁺ may act on cells. It has been proposed that extracellular NAD⁺ may modulate cellular responses by acting as a substrate for endogenous ADP-ribosyltransferases (33, 34). Five mammalian ADP-ribosyltransferases (ART1–5) have been identified (35). Interestingly, CD38 has also been shown to possess ADP-ribosyl transferase activity (36). The ADP-ribosyltransferases are structurally and functionally related to cholera and pertussis toxins, which interfere

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5 R. Zamora, N. Azhar, R. Namas, M. R. Metukuri, T. Clermont, C. Gladstone, R. A. Namas, L. Hermus, C. Megas, G. Constantine, T. R. Billiar, M. P. Fink, and Y. Vodovotz, manuscript in preparation.
with signal transduction in human host cells by ADP-ribosylating regulatory G-proteins (3). Several structurally unrelated inhibitors of ADP-ribosyltransferase were found to inhibit LPS-induced production of reactive oxygen intermediates and TNF-α by human mononuclear cells (37, 38).

Our results demonstrate that mouse macrophage-like cells treated with NAD⁺ exhibit elevated expression of both active and latent TGF-β1, with a biphasic time course that also depends on the specific concentration of NAD⁺. In an attempt to discern the mechanisms responsible for this complexity, we utilized both data-driven and mechanistic computational modeling. Complex, multidimensional features of inflammation and associated processes (e.g. apoptosis) have been elucidated using such computational methods, for example, suggesting that the regulation of the transcription factor NF-κB involves oscillations in the expression of its inhibitor, IκB (39), and that signaling for apoptosis can be reduced to two principal components (40). An initial statistical analysis suggested that the interactions among NAD⁺, latent TGF-β1, and active TGF-β1 were nonlinear. A series of nonlinear mechanistic mathematical models of increasing complexity were generated in an attempt to determine the mechanisms responsible for these nonlinear interactions. When combined with our biochemical data, our models suggested that NAD⁺ mediates its complex effects on

FIGURE 6. Complex dose- and time-course of the expression of active and latent TGF-β1 in RAW 264.7 cells treated with NAD⁺. RAW 264.7 cells were treated with 0, 10, 100, or 1000 μM NAD⁺ for 0–24 h as indicated and subsequently immunostained for active (panel A) or latent (panel D) TGF-β1 as described under “Experimental Procedures.” The results are the mean ± S.E. of 5–7 experiments. Panel A, *, p < 0.05 versus 0 μM NAD⁺; #, p < 0.05 versus 100 μM NAD⁺ at 1 h; panel D, *, p < 0.05 versus 0 μM NAD⁺; #, p < 0.05 versus 100 μM NAD⁺ at given time point; †, p < 0.05 versus 10 μM NAD⁺ at 1 h; $, p < 0.05 versus 100 μM NAD⁺ at 6 h, analyzed by one-way analysis of variance followed by the Fisher LSD method. Values generated by a statistical model and a nonlinear ODE model are shown in panels B and C for active TGF-β1 and in panels E–F for latent TGF-β1. Time points in boxes (panels B–F) represent good concordance with the experimental data.
Effects of NAD$^+$ on Macrophage TGF-β1

TGF-β1 via cADPR, secondary to stimulation of Ca$^{2+}$ fluxes. Our results do not rule out a role of ADP-ribosyltransferases in the effect of NAD$^+$ on TGF-β1 however. In fact, the inability of our mechanistic mathematical model, which does not incorporate the ADP-ribosyltransferases, to fully predict the multiphasic behavior of TGF-β1 in response to NAD$^+$ may suggest that we have not fully accounted for all the relevant biological mechanisms in modeling this process.

### TABLE 1

| Description of parameter values used for the ODE model |
|-------------------------------------------------------|
| Parameter | Value | Description |
| $k_1$ | 30 | Rate of NAD$^+$ consumption to form cADPR |
| $k_2$ | 10 | Rate of formation of X$_1$ |
| $k_3$ | 1 | Rate of formation of TGF-β1 mRNA from X$_2$ |
| $k_{NAD}$ | 1 | Degradation rate of NAD$^+$ |
| $k_{Ca}$ | 1000 | Degradation rate of Ca$^{2+}$ |
| $k_{Lat}$ | 56 | Degradation rate of Latent TGF-β1 |
| $k_{Adv}$ | 12 | Degradation rate of Active TGF-β1 |
| $k_{End}$ | 1.3 | Degradation rate of X$_1$ and X$_2$ |
| $k_{Tgf}$ | 3.9 | Degradation rate of TGF-β1 mRNA |
| $k_{Act}$ | 1500 | Rate of activation of TGF-β1 |
| $k_{Adv}$ | 15 | Rate of translation of TGF-β1 mRNA |
| $k_X$ | 10 | Rate of formation of X$_3$ |
| $k_{neg}$ | 4.5 | Rate of Ca$^{2+}$-mediated degradation of TGF-β1 mRNA |
| $\beta_c$ | 12 | Basal rate of Ca$^{2+}$ production in cell |
| $\beta_m$ | 2 | Basal rate of TGF-β1 mRNA production in cell |
| $\alpha$ | 1 | Hill coefficient for X$_1$ production |
| $C_{thresh}$ | 1000 | Threshold for activation of Ca$^{2+}$-mediated inhibition |

In our studies we found that blocking Ca$^{2+}$ with the Ca$^{2+}$ chelator BAPTA inhibited the activation of TGF-β1 by NAD$^+$ or cADPR and that treatment with the Ca$^{2+}$ agonists ionomycin and bradykinin both induced increased expression as well as activation of TGF-β1. These studies suggest that modulation of Ca$^{2+}$ is a central mechanism responsible for the elevation of TGF-β1. However, in multiple attempts we could not reproducibly demonstrate Ca$^{2+}$ fluxes in RAW 264.7 cells (data not shown). Interestingly, whereas many cell lines exhibit Ca$^{2+}$ fluxes in response to extracellular NAD$^+$ (41, 42), there are no published reports of ryanodine-sensitive Ca$^{2+}$ flux measurements in RAW 264.7 cells. It is, therefore, unclear if the effects of NAD$^+$ and cADPR require the activation of ryanodine-sensitive Ca$^{2+}$ channels. Moreover, our data suggest that the effects of NAD$^+$ on TGF-β1 requires L-type Ca$^{2+}$ channels, although we have not defined the specific channels involved.

There are several limitations to our study. The first is that although the main finding of NAD$^+$/cADPR-induced induction of TGF-β1 was shown both in RAW264.7 cells and in primary macrophages, the detailed time- and dose-curve experiments were carried out only in the former. We note that the extensive time- and dose-curve studies performed over many repeats would have been extremely cumbersome in primary cells. Moreover, we expect to have observed much greater variability in the biological responses studied. Another limitation is the extensive use of semiquantitative immunocytochemistry.

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**FIGURE 7.** Validation of computational models of the effects of NAD$^+$ on active and latent TGF-β1. The predictive capacity of the statistical model created with the data in Fig. 5 was tested by treating RAW 264.7 cells with 0, 10, 100, or 1000 μM NAD$^+$ for 16 h (a time point not used in the construction of the model). The cultures were stained for the presence of active (panel A) or latent (panel B) TGF-β1 as described under the “Experimental Procedures.” Open bars show actual data (panel A, n = 6; *, p < 0.05 versus 0, 10, and 1000 μM NAD$^+$; **, p < 0.05 versus 0 and 10 μM NAD$^+$; panel B, n = 6, *, p < 0.05 versus 0, 10, and 1000 μM NAD$^+$; **, p < 0.05 versus 0 and 10 μM NAD$^+$; #, p < 0.05 versus 0 μM NAD$^+$). In both panels gray bars show predictions using a statistical model, and black bars show predictions using an ODE model as described under “Experimental Procedures.” Panel C, total RNA was isolated from untreated (control) or RAW 264.7 cells treated with 10, 100, or 1000 μM NAD$^+$ for 21 h followed by Northern blotting and analysis for TGF-β1 mRNA as described under “Experimental Procedures.” The relative amount of mRNA is presented as the ratio of mRNA to 18S RNA (a representative blot from three independent experiments is shown in the inset).
Effects of NAD<sup>+</sup> on Macrophage TGF-β1

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Effects of NAD\textsuperscript{+} on Macrophage TGF-β1

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