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# NAD$^+$ dependent deacetylase Sirtuin 5 rescues the innate inflammatory response of endotoxin tolerant macrophages by promoting acetylation of p65

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ABSTRACT

The induction and persistence of a hypo-inflammatory and immunosuppressive state in severe sepsis is commonly associated with increased risks of secondary infections and mortality. Toll-like receptor (TLR)-triggered inflammatory response of macrophages/monocytes plays an important role in determining the outcome of hyper-inflammation during the acute phase and the hypo-inflammation during immunosuppressive phase of sepsis. However, the mechanisms for controlling hypo-inflammatory response in endotoxin tolerant macrophages remain to be fully understood. Considering that metabolic control of inflammation is an emerging field and the balance between AMP/ATP and oxidized NAD$^+$/reduced NADH is associated with inflammation and metabolism, we analyzed the level of NAD$^+$ in TLR-triggered innate inflammatory response, and found that the decreased level of NAD$^+$ was significantly related to the increased inflammatory cytokine production both *in vivo* and *in vitro*. By screening the expression and function of NAD$^+$ dependent type III deacetylase Sirtuin family members, we found that SIRT5 and SIRT1/2 had opposite expression patterns and functions in macrophages. *SIRT5* deficiency decreased TLR-triggered inflammation in both acute and immunosuppressive phases of sepsis. Interestingly, cytoplasmic SIRT5 counteracted the inhibitory effects of SIRT2 and enhanced the innate inflammatory responses in macrophages and even in endotoxin-tolerant macrophages by promoting acetylation of p65 and activation of NF-κB pathway. Mechanistically, SIRT5 competed with SIRT2 to interact with NF-κB p65, in a deacetylase activity-independent way, to block the deacetylation of p65 by SIRT2, which consequently led to increased acetylation of p65 and the activation of NF-κB pathway and its downstream cytokines. Our study discovered the new functions of different Sirtuin members in sepsis, indicating that targeting of Sirtuin family members at different sepsis phases can be helpful to precisely control the progression of sepsis.



## 1. Introduction

During severe sepsis, the exaggerated inflammatory response leads to multi-organ failure and systemic inflammatory response syndrome (SIRS), characterized as an early hyper-inflammatory status of acute phase, then followed by shifting to a hypo-inflammatory status of immunosuppressive phase. The later phase is called compensatory anti-inflammatory response syndrome (CARS) [1]. The presence and persistence of a hypo-inflammatory status in severe sepsis is associated with an increased risk of secondary infections and mortality. Pathologically, the overactivation of Toll-like receptor (TLR) with a hyper-inflammatory response during the acute phase and the failure of TLR activation with a hypo-inflammatory response upon secondary infections, also called endotoxin tolerance, are both important mechanism of sepsis [2,3]. Secondary infections result in repeated cycles of hyper and hypo-inflammatory phases, which further complicates the septic process. By far, most studies that investigate

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positive or negative regulatory mechanisms of TLR signaling [4–9] focus only on one phase of sepsis, especially the acute phase. Clinically, many efforts to block the initial pro-inflammatory phase, such as applying Anakinra (recombinant human IL-1Ra) in early phase of septic patients had been made. But this strategy failed to show any survival benefit, which may result from the aggravation of hypo-inflammation during immunosuppressive phase of sepsis after Anakinra treatment [10]. These clinical trials thus raise a question that whether amelioration of initial hyper-inflammation either by blocking pro-inflammatory cytokines or by inhibiting TLR signaling will aggravate the following hypo-inflammation, which needs to be addressed. Another important question remains to be addressed is that the switch from the hyper-inflammatory phase to the hypo-inflammatory phase can hardly be identified in clinically septic patients because of the repeated or mixed infection from either host commensal microbiome or invading environment microbial. Thus, these two phases of sepsis are tightly related and determine the outcome of the patients together. New insight into the inflammatory responses in both phases of sepsis and identifying new markers to discriminate these two phases is of important significance to precisely designing therapeutic strategies for sepsis.

Cellular bioenergy balance between AMP/ATP and $NAD^+$/NADH is associated with inflammation and metabolism, including intracellular signaling transduction, nuclear transcription factors activation, and chromatin structure status [11]. Elevated ATP and peroxisome proliferator-activated receptor (PPAR) $\gamma$ coactivator 1-$\alpha$ mRNA expressions in muscle biopsies of sepsis patients are associated with better survival [12], whereas mitochondrial swelling and energy shortage have been hypothesized to be an underlying cause of organ dysfunction [13,14]. Recently, a shift from oxidative phosphorylation to aerobic glycolysis was found to be important for initial activation of host defense upon acute inflammation [15]. In an endotoxin-tolerance model, the metabolism switches from glycolysis to fatty acid oxidation in monocytes to resolve acute inflammation [16]. This catabolic shift also supports the differentiation of M2-like macrophages, which are also critical cells for hypo-inflammation responses [17]. Above researches indicate the importance of efficient energy supply in defending against microbial pathogens. However, glucose from nutritional supplementation was recently found to be detrimental for anti-bacterial defense, whereas be required for anti-viral immune response [18]. Efficient energy supply will decrease the ratios of AMP/ATP and $NAD^+$/NADH. Whether the balance between AMP/ATP and $NAD^+$/NADH from glycolysis or fatty acid oxidation metabolism is related to hyper- or hypo-inflammatory status of sepsis needs to be addressed.

Sirtuins (SIRT), first discovered in yeast as $NAD^+$ dependent epigenetic and metabolic regulators, have comparable activities in human physiology and disease [19]. Mounting evidence supports that the seven-member mammalian Sirtuin family (SIRT1–7) guard cellular homeostasis by sensing bioenergy needs and by making alterations in the cell nutrients [20,21]. Sirtuins play a critical role in restoring homeostasis during stress responses. SIRT1 and SIRT6 proteins coordinate a switch from glucose to fatty acid oxidation during the acute inflammatory response in THP-1 monocytes [16]. SIRT1/2 was found to deacetylate p65 and deacetylate chromatin histone H4, which suppressed inflammation [22,23]. Increasing data show that $NAD^+$ levels and SIRT expression levels are persistently reduced in specific tissue during chronic inflammation diseases, such as brain of Alzheimer's disease and arteria of atherosclerosis [20]. A comprehensive investigation of cellular metabolism in macrophages, such as the ratios of $NAD^+$/NADH and the dynamic expression of Sirtuin family members during the switch from the hyper-inflammatory status of acute phase to the hypo-inflammatory status of immunosuppressive phase of sepsis and their effects on immunological function and outcome, will contribute to a better understanding of sepsis pathology.

By dynamically observing the level of $NAD^+$ upon inflammation, we found that $NAD^+$ production was significantly related with inflammatory cytokine production during sepsis *in vivo* and *in vitro*. Accordingly, the expression the Sirtuin family members also changed dynamically. Function screening revealed SIRT5 had opposite function on TLR-induced response against SIRT1/2. *SIRT5* deficiency decreased TLR-triggered inflammation in both hyper-inflammatory/acute and hypo-inflammatory/immunosuppressive phases. SIRT5 competed with SIRT2 to interact with p65 subunit and then blocked the deacetylation of p65 by SIRT2 in an enzymatic activity independent way. Our results outline a new metabolic pathway in both hyper-inflammatory/acute and hypo-inflammatory/immunosuppressive phases of sepsis, which indicates that targeting Sirtuin family at different phases of sepsis will be a promising strategy for precise control of sepsis.

## 2. Materials and methods

### 2.1. Mice

*SIRT5*$^{+/-}$ heterozygote mice were from The Jackson Laboratory (B6; 129-SIRT5tm1Fwa/J; Stock Number:012757) and bred in pathogen-free conditions. *SIRT5*$^{+/+}$ and *SIRT5*$^{-/-}$ homozygote littermates were derived from the *SIRT5*$^{+/-}$ heterozygote mice mating with each other. 6-8-week-old littermate mice were used in the experiments (body weight and sexuality balanced). All animal experiments were performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University, Shanghai.

### 2.2. Reagents

LPS (O111:B4), poly (I:C) and CpG ODN were from Sigma-Aldrich as described previously [4,24]. $NAD^+$ was from MCE Express. Antibodies specific for V5-tag, Myc-tag, Flag-tag (HRP conjugated) and the agaroses used in immunoprecipitations were from Abcam Inc. Abs specific for actin, SIRT5, SIRT2, SIRT1, HSP60, HSP90, Lam A/C, GAPDH, F4/80 and p65, and phospho-specific Abs against p65 (Ser536), P38 (Thr180/Tyr182), ERK (Thr202/Tyr204), JNK (Thr183/Tyr185) and Acetyl-p65 (Lys310) were from Cell Signaling Technology (Beverly, MA). HRP-conjugated second antibody (TrueBlot) was from eBioscience.

### 2.3. $NAD^+$ extraction and colorimetric $NAD^+$ assay

$NAD^+$ extraction and evaluation were performed using EnzyChrom $NAD^+$/NADH Assay kit from BioAssay System according to the manufacturer's instructions. $6 \times 10^5$ cells or 20 mg tissues were collected to test the $NAD^+$ level.

### 2.4. Nuclear and cytoplasmic extraction

Cellular nuclear and cytoplasmic extraction were performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents from Invitrogen according to the manufacturer's instructions.

### 2.5. Mitochondria isolation

Cellular mitochondria isolation from cytoplasm was performed using Mitochondria Isolation Kit for cultured cells from Invitrogen according to the manufacturer's instructions.