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Extracellular NAD is a regulator for FcγR-mediated phagocytosis in murine macrophages

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Abstract

NAD is available in the extracellular environment and elicits immune modulation such as T cell apoptosis by being used as the substrate of cell surface ADP-ribosyl transferase. However, it is unclear whether extracellular NAD affects function of macrophages expressing cell surface ADP-ribosyl transferase. Here we show that extracellular NAD enhances Fcγ receptor (FcγR)-mediated phagocytosis in J774A.1 macrophages via the conversion into cyclic ADP-ribose (cADPR), a potent calcium mobilizer, by CD38, an ADP-ribosyl cyclase. Extracellular NAD increased the phagocytosis of IgG-coated sheep red blood cells (IgG-SRBC) in J774A.1 macrophages, which was completely abolished by pretreatment of 8-bromo-cADPR, an antagonist of cADPR, or CD38 knockdown. Extracellular NAD increased basal intracellular Ca^{2+} concentration, which also was abolished by pretreatment of 8-bromo-cADPR or CD38 knockdown. Moreover, the chelation of intracellular calcium abolished NAD-induced enhancement of phagocytosis of IgG-SRBC. Our results suggest that extracellular NAD act as a regulator for FcγR-mediated phagocytosis in macrophages.

Keywords: Macrophages; Fcγ receptor; Phagocytosis; NAD; Cyclic ADP-ribose

NAD is a coenzyme with critical role in energy metabolism and electron transfer. Most of the cellular NAD^+ is stored in the mitochondria for the utilization on metabolic purposes. NAD^+ in the cytoplasm acts as a cofactor of various enzymes such as various NAD^+-dependent dehydrogenases whereas NAD in the nucleus plays cell signaling roles as a substrate for two families of nuclear enzymes, i.e., poly-ADP-ribosyl polymerases and the sirtuin family of NAD-dependant lysine deacetylases, both of which is involved in coordination of DNA repair, regulation of transcription levels and control of progression towards apoptosis [1–3]. Recently it has been demonstrated that NAD^+ plays an important role also in the extracellular environment, i.e., as substrates for a variety of nucleotide-metabolizing ectoenzymes such as ADP-ribosyl cyclase, NAD glycohydrolase and ADP-ribosyl transferase (ART). NAD in the extracellular environment is available through passive release from dying cells or active secretion through connexin 43 hemichannel in living cells [4,5].

NAD in the extracellular environment has been demonstrated to act as an immune modulator in T cells [6–9].

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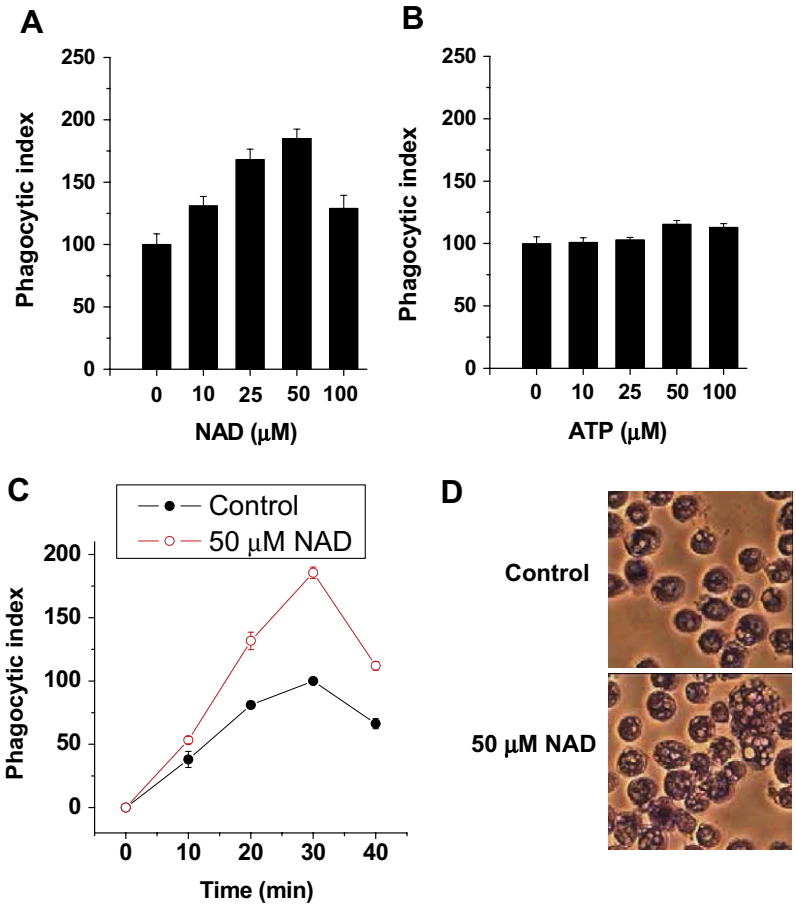

Fig. 1. The effect of extracellular NAD and ATP on FcγR-mediated phagocytosis in J774A.1 cells. J774A.1 cells were pretreated with the indicated concentrations of NAD (A) and ATP (B) for 30 min at 37 °C with 5% CO_2, after which they were subjected to IgG-SRBC in fresh medium at a target-to-effector ratio equal to 200:1 for 30 min at 37 °C. Nonengulfed SRBCs were lysed by water shock and the cells were fixed and stained with Wright Giemsa staining before the phagocytic index was counted. Columns indicate phagocytic index of J774A.1 cells (mean ± SD). (C) Time-courses of FcγR-mediated phagocytosis with or without 50 μM NAD. J774A.1 cells were pretreated with or without 50 μM NAD for 30 min at 37 °C with 5% CO_2, after which they were subjected to IgG-SRBC in fresh medium at a target-to-effector ratio equal to 200:1 for the indicated times at 37 °C. Data indicates mean ± SD. (D) An example for light microscopy showing NAD-induced enhancement of IgG-SRBC phagocytosis.

Extracellular NAD induces apoptosis of T cells via ART-mediated ADP-ribosylation of cell surface proteins. In naive T cells, extracellular NAD induces apoptosis of naive mouse T cells through triggering P2X7 purinergic receptor by ART2-mediated ADP-ribosylation [9]. In anti-CD3 antibody-activated T cells, extracellular NAD induces apoptosis through ADP-ribosylation of CD38, an ADP-ribosyl cyclase [7]. Interestingly, apoptosis is observed with NAD concentrations as low as 1 micromolar, which is close to the concentration in mouse serum [7,10]. These findings indicate that extracellular NAD-induced modulation of cell function is a physiologically relevant event.

Macrophages are immune cells with specialized capacity for cell migration and phagocytosis of complement and immunoglobulin-opsonized pathogens. Phagocytosis of immunoglobulin G (IgG) opsonized particles is mediated via Fcγ receptors (FcγRs) [11]. Murine macrophages express two classes of activating FcγRs, FcγRI and FcγRIIIa, which are associated with the low molecular weight γ-subunit bearing a tyrosine-based activation motif [11]. Upon encountering an immune complex, FcγRs on macrophages are clustered and the immunoreceptor tyrosine-based activation motifs (ITAMs) are phosphorylated by the membrane-associated Src kinases. The phosphorylated ITAMs recruit several signaling enzymes and adapter-enzyme complexes, activating a cascade of signaling events that culminates in phagocytosis and generation of inflammatory mediators [12,13].

Paracrinal secretion of cytokines such as interferon-γ, GM-CSF, interleukin-18 and interleukin 12 modulates FcγR-mediated phagocytosis in macrophages [14,15]. NAD also acts like a paracrinal cytokine in a certain condition in which paracrinal secretion of NAD increases intracellular calcium in the neighboring cells and proliferates the cells through the conversion of NAD into cyclic ADP-ribose (cADPR) by CD38 [16]. Extracellular NAD is utilized as the substrate of ART, ADP-ribosylates P2X7 purinergic receptor and induces apoptosis of T cells [9]. It is possible that extracellular NAD affects FcγR-mediated phagocytosis of macrophages because macrophages

express CD38, ART and P2X7 purinergic receptor [17–19]. However, there is no report about the effect of extracellular NAD on FcγR-mediated phagocytosis. In this study we examined whether and how extracellular NAD affects FcγR-mediated phagocytosis in J774A.1 macrophage cells.

Materials and methods

Cell culture. The murine macrophage cell line J774A.1 (obtained from ATCC) was maintained at 37 °C, 5% CO_2 incubator in DMEM supplemented with 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD). The cells were passaged weekly, and cells older than 15 passages were not used.

Phagocytic assay. Phagocytic assay was performed as previously described with some modification [20,21]. J774A.1 cells were plated at a density of 1×10^5 cells per well in a 12-well plate (Costar, Corning, NY) overnight. The cells were pre-treated with various nucleotides for 30 min at 37 °C in 5% CO_2 incubator. For phagocytosis, the media was removed and replaced with DMEM containing IgG-coated sheep red blood cells (IgG-SRBC) for 30 min. IgG-SRBC was prepared by incubating sheep red blood cells with 1:10 dilution of the maximal subagglutinating titer of rabbit anti-sheep RBC IgG (ICN-Cappel, Aurora, OH). Cell surface bound SRBC were lysed with hypotonic buffer, and the cells were fixed in 3.7% paraformaldehyde solution. Phagocytosis was assessed by light microscopy. The phagocytic index was calculated as follows: phagocytic index (PI) = number of SRBCs internalized by 100 J774 cells counted in 10 random fields.

Fluorimetric determination of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$). J774A. 1 cells were incubated with 4 μM Fura-2 AM in RPMI 1640 medium containing 3% fetal bovine serum for 60 min at 37 °C. The Fura-2-loaded cells were then washed twice with HBSS. For the fluorometric measurement of Ca^{2+}, 5×10^6 cells were placed in a quartz cuvette in a thermostatically controlled cell holder at 37 °C and the cell suspension was stirred continuously. Fluorescence ratios were taken with an alternative wavelength time scanning method (dual excitation at 340 and 380 nm; emission at 510 nm) using a PTI fluorometer (Photon Technology International, Lawrenceville, NJ).

Calculation of $[Ca^{2+}]_i$. The intracellular free calcium concentration was calculated according to Eq. (1) [22].

$$[Ca^{2+}]_i = \beta K_D (R - R_{min})/(R_{max} - R) \quad (1)$$

where R is E_{340}/E_{380}; R_{min} is E_{340}/E_{380} in zero calcium; R_{max} is E_{340}/E_{380} in calcium saturated solution; β is E_{380} in zero calcium/E_{380} in calcium-saturated solution; and $K_D = 140$ nM (the dissociation constant of the dye at room temperature). To obtain the parameter values, after each experiment cells were treated with 0.1% triton X-100 and then 4 mM EGTA. At the end of this procedure, 5 mM $MnCl_2$ was added to the bath to quench the fluorescence of the dye and determine the background values.

Knockdown of CD38 using CD38 siRNA. We purchased control and CD38 siRNA from Santacruz Biotechnology (CA, USA). A total of 60 pmol of siRNA duplexes were transfected into 5×10^5 cells using Nucleofector (Amaxa Inc., Gaithersburg, MD). After 48 h of transfection, cells were prepared for examination. The knockdown of CD38 was confirmed by Western blotting using anti-CD38 antibody (Santacruz, CA, USA).

Statistical analysis. Statistical significance was determined using a two tailed Student's *t* test and one way Anova test followed by Scheffe's post hoc test.

Results and discussions

NAD enhances FcγR-mediated phagocytosis

In order to investigate the role of extracellular NAD in IgG-SRBC phagocytosis, we incubated J774A.1 cells with various concentration of NAD for 30 min, added IgG-SRBC and measured phagocytic index. The pretreatment of extracellular NAD strongly enhanced IgG-SRBC phagocytosis in J774A.1 cells (Fig. 1A). The enhancement was concentration-dependent in the range 0–50 μM, and reached a maximum at 50 μM (Fig. 1A). Recent evidences suggest that NAD stimulates P2X7 purinoreceptor by ART2-mediated ADP-ribosylation and induces T cell apoptosis [9]. It has been known that J774 cells express P2X7 purinoreceptor [23]. It is possible that NAD enhances phagocytosis of IgG-SRBC via the stimulation of P2X7 purinoreceptor. To test this, we examined the effect of ATP, a P2X7 stimulator, on IgG-SRBC phagocytosis. We incubated J774A.1 cells with various concentration of ATP for 30 min, added IgG-SRBC and measured phagocytic index. The pretreatment of extracellular ATP didn't affect IgG-SRBC phagocytosis in J774A.1 cells (Fig. 1B). As shown in a time-course data of phagocytosis (Fig. 1C), NAD-induced increase of IgG-SRBC phagocytosis appears to be due to acceleration of phagocytosis, but not, augmentation of phagocytosis ability. These data suggest that NAD enhances FcγR-mediated phagocytosis through a different pathway from P2X7 purinoreceptor.

cADPR among NAD metabolites enhances FcγR-mediated phagocytosis

Extracellular NAD serves as a substrate for cell-surface ART2 and produces free ADP-ribose (ADPR) and ADP-ribosylated protein [24]. In addition, extracellular NAD is hydrolyzed to ADPR, cADPR and nicotinamide by CD38 [25]. To examine whether these NAD metabolites mediate NAD-induced enhancement of IgG-SRBC phagocytosis, we incubated J774A.1 cells with various concentra-

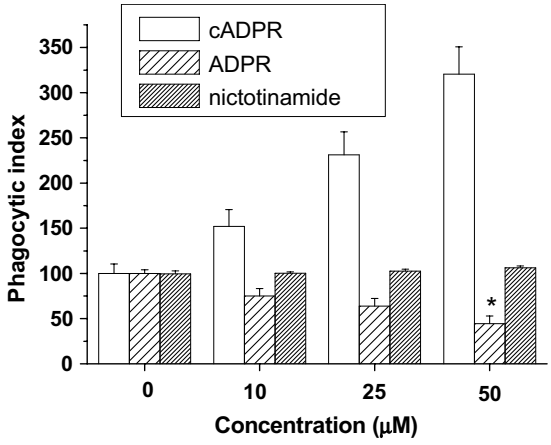

Fig. 2. The effect of extracellular cADPR, ADPR and nicotinamide on FcγR-mediated phagocytosis in J774A.1 cells. J774A.1 cells were pretreated with the indicated concentrations of cADPR, ADPR and nicotinamide for 30 min at 37 °C with 5% CO_2, after which IgG-SRBC phagocytosis was measured as described in Fig. 1 legend. Columns indicate phagocytic index of J774A.1 cells (mean ± SD). $^*p < 0.001$ versus control.

tion of cADPR, ADPR and nicotinamide for 30 min, added IgG-SRBC and measured phagocytic index. The pretreatment of extracellular ADPR decreased phagocytosis of IgG-SRBC and nicotinamide didn't affect phagocytosis of IgG-SRBC (Fig. 2). However, cADPR pretreatment enhanced IgG-SRBC phagocytosis (Fig. 2). These data suggest that NAD enhances IgG-SRBC phagocytosis through cADPR production.

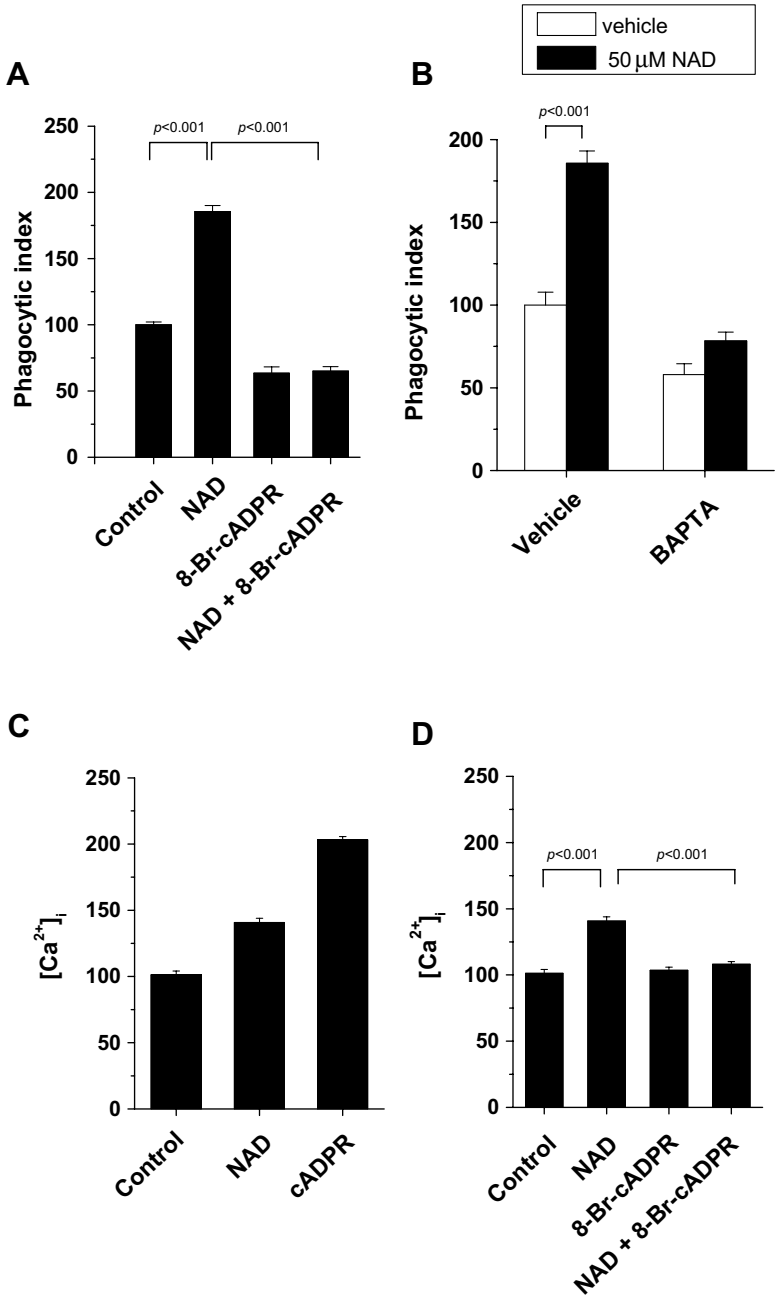

Fig. 3. NAD enhances FcγR-mediated phagocytosis through cADPR production. (A) The effect of 8-Br-cADPR on NAD-induced enhancement of IgG-SRBC phagocytosis. J774A.1 cells (5×10^6 cells) were pretreated with 50 μM NAD, 5 μM 8-Br-cADPR and 50 μM NAD plus 5 μM 8-Br-cADPR for 20 min at 37 °C with 5% CO_2, and then then IgG-SRBC phagocytosis was measured as described in Fig. 1 legend. Columns indicate phagocytic index of J774A.1 cells treated with vehicle or various reagents (mean ± SD). (B) The effect of intracellular calcium chelation on NAD-induced enhancement of FcγR-mediated phagocytosis. J774A.1 cells (5×10^6 cells) were incubated with 50 μM BAPTA for 30 min at 37 °C with 5% CO_2 and then IgG-SRBC phagocytosis was measured as described in Fig. 1 legend. Columns indicate phagocytic index of J774A.1 cells treated with vehicle or BAPTA (mean ± SD). (C) The effect of NAD and cADPR on $[Ca^{2+}]_i$. Fura 2 loaded J774A.1 cells (5×10^6 cells) were pretreated with vehicle, 50 μM NAD and 5 μM cADPR for 20 min at 37 °C with 5% CO_2, after which they were subjected to a quartz cuvette in a thermostatically controlled cell holder at 37 °C for the measurement of fluroscence ratio, as described in Materials and Methods. (D) The inhibition NAD-mediated increase in $[Ca^{2+}]_i$ by 8-Br-cADPR. Fura 2 loaded J774A.1 cells (5×10^6 cells) were pretreated with 50 μM NAD, 5 μM 8-Br-cADPR and 50 μM NAD plus 5 μM 8-Br-cADPR for 20 min at 37 °C with 5% CO_2, after which they were analysed for $[Ca^{2+}]_i$ measurement as described above. $[Ca^{2+}]_i$ was calculated from three experiments.

NAD enhances FcγR-mediated phagocytosis through cADPR production

To further confirm the possibility that NAD enhances IgG-SRBC phagocytosis through cADPR production, we tested the effect of 8-bromo-cADPR (8-Br-cADPR), an antagonist for cADPR, on IgG-SRBC phagocytosis. 8-Br-cADPR completely blocked NAD-induced enhancement of IgG-SRBC phagocytosis (Fig. 3A). cADPR is known to be an intracellular calcium-mobilizing agent [26]. Thus, we checked whether NAD enhances IgG-SRBC phagocytosis through cADPR-mediated increase in intracellular calcium. Intracellular calcium chelation with BAPTA also completely blocked NAD-induced enhancement of IgG-SRBC phagocytosis (Fig. 3B), indicating that NAD-induced enhancement of IgG-SRBC phagocytosis is dependent on intracellular calcium concentration. Indeed, extracellular NAD enhanced $[Ca^{2+}]_i$ although the extent to which NAD increases $[Ca^{2+}]_i$ is lower than cADPR (Fig. 3C), which was completely blocked by 8-Br-cADPR (Fig. 3D). These data suggest that NAD enhances IgG-SRBC phagocytosis through cADPR production and subsequent increase in $[Ca^{2+}]_i$.

CD38 mediates NAD-induced enhancement of Fcγ R-mediated phagocytosis

CD38 possesses NAD-glycohydrolase and ADP-ribosyl cyclase activities and catalyzes the cleavage of NAD into ADPR, cADPR and nicotinamide [25]. To demonstrate whether NAD-induced enhancement of IgG-SRBC phagocytosis is mediated by the ADP-ribosyl cyclase activity of CD38, we knockdown CD38 with CD38 siRNA and measured the effect of CD38 knockdown on NAD-induced increase in $[Ca^{2+}]_i$ and enhancement of IgG-SRBC phagocytosis. We confirmed the knockdown of CD38 by Western blotting (Fig. 4A). CD38 knockdown abrogated the NAD-induced $[Ca^{2+}]_i$ increase (Fig. 4B) and enhancement of

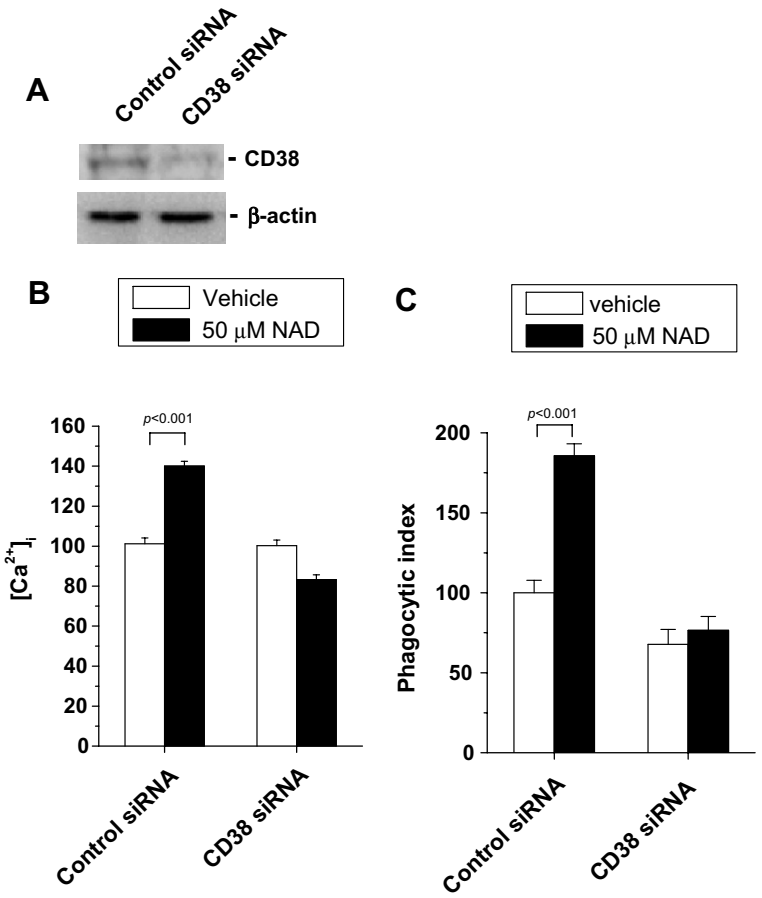

Fig. 4. CD38 mediates NAD-induced enhancement of FcγR-mediated phagocytosis. (A) The expression levels of CD38 in control or CD38 siRNA-transfected J774A.1 cells, as detected by Western blot. A total of 60 pmol of siRNA duplexes were transfected into 5×10^5 cells using Nucleofector. After 48 h of transfection, the cells were harvested and analyzed by Western blot (B) The effect of CD38 knockdown on NAD-induced increase in $[Ca^{2+}]_i$. CD38 was knockdowned as described above. The cells were pretreated with 50 μM NAD for 30 min at 37 °C with 5% CO_2, after which they were analyzed for $[Ca^{2+}]_i$ measurement as described in Fig. 3C legend. $[Ca^{2+}]_i$ was calculated from three experiments. (C) The effect of CD38 knockdown on NAD-induced enhancement of FcγR-mediated phagocytosis. CD38 was knockdowned as described above. J774A.1 cells (1×10^5 cells) were incubated with 50 μM NAD for 30 min at 37 °C with 5% CO_2 and then IgG-SRBC phagocytosis was measured as described in Fig. 1 legend. Columns indicate phagocytic index of J774A.1 cells treated with vehicle or NAD (mean ± SD).

IgG-SRBC phagocytosis (Fig. 4C), indicating the involvement of CD38 in NAD-induced enhancement of IgG-SRBC phagocytosis via $[Ca^{2+}]_i$ increase.

In this study, we demonstrated that extracellular NAD is converted to cADPR, a calcium mobilizing agent, by CD38 and induces an increase in $[Ca^{2+}]_i$, thereby regulating FcγR-mediated phagocytosis. Connexin 43 expressing cells can provide cADPR to neighboring cells and enhance their $[Ca^{2+}]_i$ levels and Ca^{2+}-dependent functions accordingly [27]. Examples of cADPR-responsive cells via paracrine processes include (i) smooth myocytes, (ii) 3T3 murine fibroblasts, (iii) hippocampal neurons, and (iv) human hemopoietic stem cells [27]. Our result shows that macrophages also response to cADPR via paracrine process and modulate their major function, phagocytosis. Our result is the first evidence showing the regulation of FcγR-mediated phagocytosis by extracellular NAD/CD38 system in murine macrophages. Taken together, extracellular NAD is a regulator for FcγR-mediated phagocytosis in murine macrophages.

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