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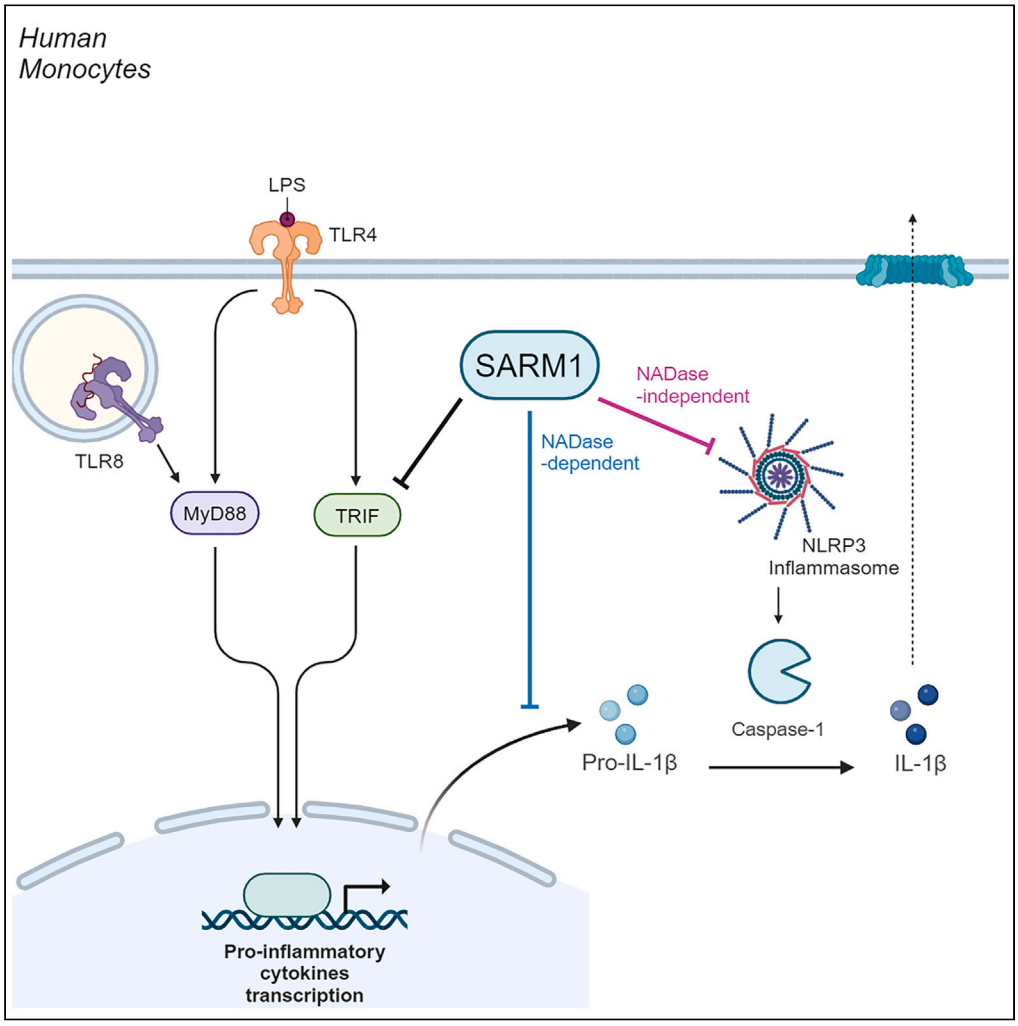

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Highlights

Human SARM1 negatively regulates pro-inflammatory cytokines in monocytes

Regulation of TLR4-stimulated TNF mRNA is independent of SARM1 NADase

Inhibition of inflammasome activation by SARM1 is NADase-independent

SARM1 inhibits IL-1β secretion via NADase-dependent and -independent mechanisms

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SARM1 regulates pro-inflammatory cytokine expression in human monocytes by NADase-dependent and -independent mechanisms

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SUMMARY

SARM1 is a Toll-IL-1 receptor (TIR) domain-containing protein with roles in innate immunity and neuronal death in diverse organisms. Unlike other innate immune TIR proteins that function as adaptors for Toll-like receptors (TLRs), SARM1 has NADase activity, and this activity regulates murine neuronal cell death. However, whether human SARM1, and its NADase activity, are involved in innate immune regulation remains unclear. Here, we show that human SARM1 regulates proinflammatory cytokine expression in both an NADase-dependent and -independent manner in monocytes. SARM1 negatively regulated TLR4-dependent TNF mRNA induction independently of its NADase activity. In contrast, SARM1 inhibited IL-1β secretion through both NADase-dependent inhibition of pro-IL-1β expression, and NADase-independent suppression of the NLRP3 inflammasome and hence processing of pro-IL-1β to mature IL-1β. Our study reveals multiple mechanisms whereby SARM1 regulates pro-inflammatory cytokines in human monocytes and shows, compared to other mammalian TIR proteins, a distinct NADase-dependent role for SARM1 in innate immunity.

INTRODUCTION

The inflammatory response protects our bodies from infection[1] or diseases, however, it also initiates pathogenic, autoimmune diseases.[2] Therefore, it is important to understand the cellular mechanisms underpinning inflammation. Expression and secretion of pro-inflammatory cytokines such as tumor necrosis factor α (TNF) and interleukin-1 β (IL-1β) from myeloid cells are key events that initiate inflammation, however how exactly these events are controlled to regulate inflammation remains unclear. Cytokines such as TNF are produced following transcriptional induction as a result of intracellular signaling from the detection of pathogens and/or host-derived danger signals by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs).[2,3] Such signaling also results in transcription of IL-1β, but in that case, pro-IL-1β is produced which then needs to be matured by its cleavage by caspase 1. Assembly of inflammasomes after infection or injury recruits the inflammasome adaptor proteins ASC, which then causes caspase-1 recruitment and activation. As well as maturing IL-1β into a secretable form, inflammasome-activated caspase-1 also cleaves gasdermin D (GSDMD) leading to GSDMD oligomerization and subsequent membrane pore formation. Because IL-1β lacks a secretion signal, the pore formed by GSDMD is required for IL-1β to be secreted from the cells.[2,4] Thus IL-1β requires 2 steps to be secreted, not only transcriptional regulation by TLRs but inflammasome activation.

Sterile alpha and HEAT/Armadillo motif-containing protein 1 (SARM1) is a member of the Toll/IL-1R (TIR) domain protein family[5] and is highly conserved in evolution with a role in innate immunity in diverse organisms.[6] Mammalian TIR proteins, such as myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor inducing interferon-β (TRIF) are known to positively mediate TLR signal transduction through TIR-TIR homotypic interactions.[2] In contrast, SARM1 negatively regulates TLR signaling through direct interaction with TRIF.[7] Another study showed that SARM1 inhibits MyD88-mediated activation of the transcription factor AP-1.[8] In addition, we recently discovered that SARM1 inhibits the NLR family pyrin domain containing 3 (NLRP3) inflammasome, in mouse bone-marrow-derived macrophages (BMDMs), by disrupting the interaction of NLRP3 with ASC, leading to reduced caspase-1 activation and IL-1β secretion.[9] Also, others reported that SARM1 is recruited by the capsaicin receptor, transient receptor potential vanilloid 1 (TRPV1) to restrain NF-κB-mediated inflammatory hepatic stellate cell activation.[10] Together these reports suggest that SARM1 may play an anti-inflammatory role. However, much of the work on SARM1 has been done in mice and mouse cells and the detailed role of human SARM1 in innate immunity, especially in human myeloid cells, remains unclear.

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A major discovery was that the TIR domain of SARM1 has NADase enzymatic activity, which is unique among mammalian TIR proteins, and that this activity is required for SARM1-dependent axon degeneration in mouse neurons.[11,12] Nicotinamide adenine dinucleotide (NAD^+) is a cofactor that plays a central role in aerobic respiration and is also utilised by many NAD^+-dependent enzymes which are crucial to cell metabolism, inflammatory responses and cell death.[13] NADases such as SARM1 have adenosine diphosphate (ADP)-ribosyl cyclase activity, generating nicotinamide (NAM) and cyclic ADP-ribose (cADPR) from NAD^+.[12] cADPR, a unique byproduct produced by NADases, is a key Ca^{2+} mobilizing agent.[14] As well as a TIR domain, SARM1 has both sterile alpha and armadillo motifs (SAMs and ARMS respectively). SARM1 proteins form an octamer by homotypic protein interactions via SAM domains[15,16] and in general, the TIR domains interact with the ARM domain to avoid dimerization of TIR domains which keeps the SARM1 NADase inactive.[17–19] Following an axonal injury in neurons, loss of nicotinamide mononucleotide adenylyltransferase 2 (NMNAT2) leads to an increase in the NMN/NAD^+ ratio in the cell which stimulates dimerization of SARM1 TIR domains within the octamer, leading to NAD^+ depletion that triggers axonal degeneration.[20] In the absence of SARM1, mouse neurons are protected from axon degeneration induced by various insults, such as vincristine,[21] bortezomib,[21] rotenone[22] and cisplatin,[23] which identified SARM1 as a central executor of the axon degeneration response.

Although the SARM1 TIR domain uniquely has NADase activity among mammalian TIR proteins, SARM1 orthologs in various organisms are also NADases.[12,14] Like mammalian SARM1, orthologs in lower organisms can also both drive axon degeneration and regulate innate immune signaling.[16,24–30] In some cases, it has been shown that the NADase activity of the TIR domain is essential for SARM1 ortholog immune functions.[27,31] However, no studies examined if mammalian myeloid cells require SARM1 NADase activity for innate immune functions.

Here we show that human SARM1 regulates cytokine expression in myeloid cells by both NADase-dependent and -independent mechanisms. We found that SARM1 negatively regulates TLR4-dependent TNF secretion in human blood monocytes, and that SARM1-deficient monocytes generated by CRISPR/Cas9 display enhanced TNF and IL-1β secretion after TLR4 stimulation. By generating human monocytes expressing either wild type SARM1 protein, or SARM1 with a point mutation in the NADase active site, we show that SARM1 inhibition of TLR4-stimulated TNF mRNA induction is NADase-independent, while SARM1 regulates IL-1β secretion through both NADase-dependent and -independent mechanisms, since suppression of the NLRP3 inflammasome by SARM1 was NADase-independent, while inhibition of pro-IL-1β protein expression by SARM1 was NADase-dependent. These findings reveal multiple mechanisms whereby human SARM1 regulates pro-inflammatory cytokines in myeloid cells and show a role for the NADase activity of SARM1 in innate immunity, distinct from other mammalian TIR proteins.

RESULTS

SARM1 negatively regulates TLR4-dependent TNF secretion in primary human monocytes

To study the function of SARM1 in human myeloid cells, we first considered primary blood monocytes. We tested siRNA knockdown of SARM1 in $CD14^+$ monocytes and confirmed the reduction of SARM1 mRNA levels post-siRNA transfection by quantitative PCR (Figure S1A), which translated into a clear but variable reduction of SARM1 protein expression across the 7 donors that we used for this study (Figure S1B). Quantification of immunoblots showed that SARM1 siRNA treatment led to a reduction of between 9% and 57% protein expression compared to cells derived from the same donor transfected with non-targeting siRNA (Figure S1B). We assessed the effect of SARM1 protein knockdown on TNF and CCL5 secretion following TLR4 stimulation with LPS and TLR8 stimulation with CL075. We found that TNF expression was increased modestly following LPS stimulation ($p = 0.06$), but not CL075 stimulation, in SARM1-knockdown cells (Figure S1C). This was consistent with our previous report showing negative regulation of TLR4 signaling by SARM1 due to antagonism of TRIF,[7] an adaptor protein used by TLR4 but not TLR8.[5] Indeed, there was a strong negative correlation between the variable endogenous SARM1 expression due simply to human donor variation and TNF secretion in $CD14^+$ monocytes (Figure S1D, $r = -0.67$). Due to the variable effect of the SARM1 siRNA on different donor cells, we also directly correlated the expression of SARM1 after siRNA treatment to TNF secretion for different donors, and this showed a strong correlation between SARM1 reduction and TNF increase (Figure S1E, $r = 0.74$). The effects of SARM1 knockdown were more modest on secretion of the chemokine CCL5[7] than were seen for TNF, since CCL5 expression was hardly affected by SARM1 knockdown in human primary monocytes stimulated with either LPS or CL075 (Figure S1F) with a lower correlation coefficient between SARM1 protein reduction and CCL5 increase for individual donors (Figure S1G, $r = 0.43$). These results indicated that that SARM1 negatively regulates TLR4-dependent TNF secretion in blood monocytes from human donors, but also revealed the challenges of using siRNA knock-down of SARM1 in primary human cells to clarify SARM1 function.

SARM1-deficient monocytes display enhanced TNF and IL-1β secretion after lipopolysaccharide stimulation

To further explore the role of human SARM1 in monocytes, and to circumvent the problem of getting only partial knockdown of SARM1 with siRNA in primary cells, we decided to focus on human monocyte cell lines. SARM1 is known to be robustly expressed in HEK293T cells[32] and in neurons,[33,34] but less is known about expression in monocyte cell lines. We measured SARM1 mRNA expression in a variety of cell lines and normalized this to human primary $CD14^+$ monocytes. As expected, SH-SY5Y, a human neuronal cell line, showed the strongest expression of SARM1 mRNA, while SARM1 mRNA was also easily detectable in HEK293T cells (Figure 1A). We found that BLaER1 cells, which can be *trans*-differentiated into monocytes to be used as a model to study immune responses,[35] expressed similar levels of SARM1 mRNA to HEK293T cells, while THP-1 cells which are commonly used as a human macrophage-like model following PMA differentiation showed much less expression (Figure 1A). For this reason, we decided to establish SARM1-deficient cells by the CRISPR/Cas9 method in BLaER1 cells (Figure S2). Immunoblot analysis showed that SARM1 protein expression was clearly observed in BLaER1, HEK293T and SH-SY5Y cells but not

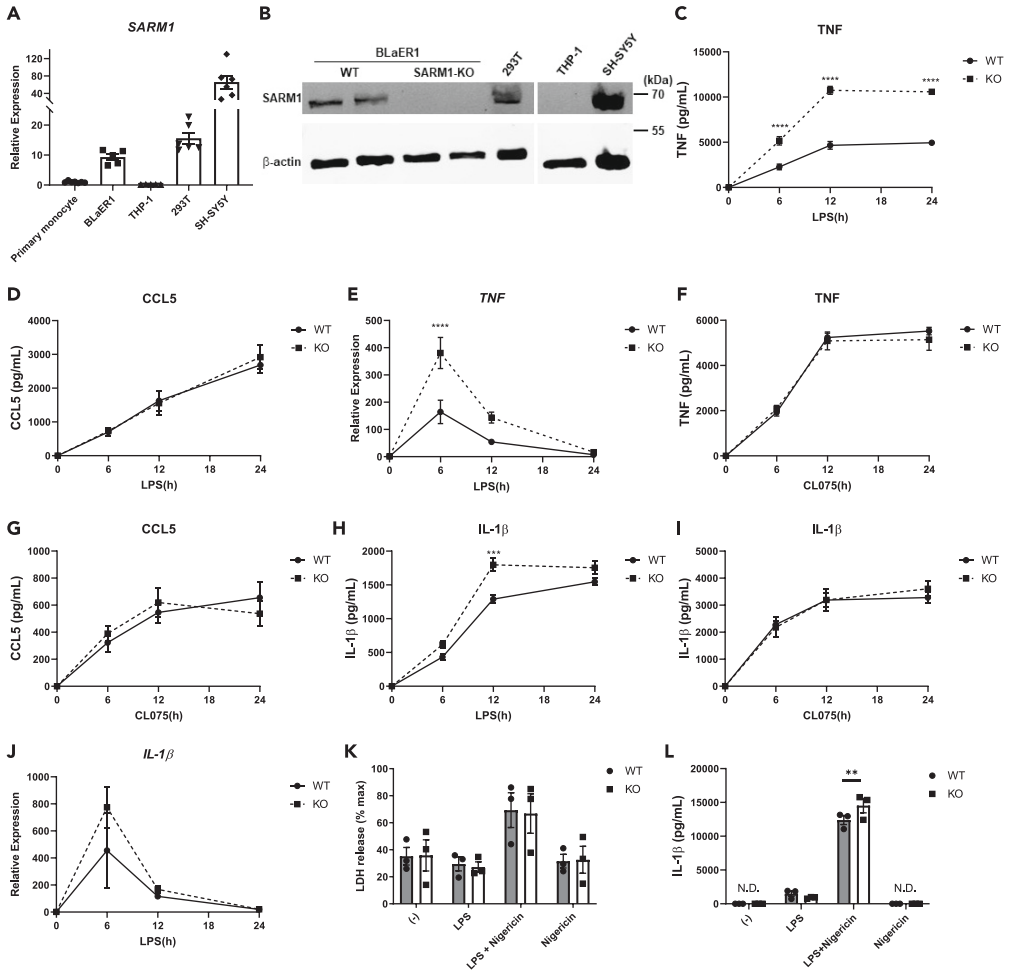

Figure 1. SARM1-deficient monocytes display enhanced TNF and IL-1β secretion after LPS stimulation

(A) Relative expression of *SARM1* mRNA was examined by qRT-PCR, normalised to primary monocytes. Data shown are mean ± SEM (bar chart) and each dot represents one donor (primary monocytes, 7 donors) or independent experiments (BLaER1 and THP-1, $n = 5$; 293T and SH-SY5Y: $n = 6$).

(B) Immunoblot of SARM1 protein in various cell lines. Each image is cropped from the same membrane. Endogenous SARM1 expression was observed in BLaER1, 293T and SH-SY5Y cells but not in THP-1 cells. Also, BLaER1 SARM1 KO cells show no detectable SARM1 protein. Representative of 3 experiments.

(C–J) BLaER1 control WT and SARM1 KO cells were differentiated into monocyte for 7 days, then stimulated with LPS (C–E and H) or CL075 (F, G, I, and J) for the indicated time (hours). Supernatants were assayed for TNF (C and F), CCL5 (D and G) and IL-1β (H and I) protein by ELISA. Relative TNF (E) and IL-1β (J) mRNA were assessed by qRT-PCR. Data shown are mean ± SEM of 3 independent experiments, each performed in triplicate.

(K and L) BLaER1 monocytes were stimulated with LPS for 4 h, following nigericin stimulation for 1 h. LDH release (K) was measured to assess pyroptosis and IL-1β secretion in the supernatant (L) was assessed by ELISA. Data shown are mean ± SEM of 3 independent experiments and each dot represents an independent experiment. Data were tested with a two-way ANOVA; Šídák's multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. See also Figure S2.

in THP-1 cells, consistent with the mRNA results, and also confirmed the absence of detectable SARM1 protein expression in SARM1-KO BLaER1 clones (Figure 1B). Following LPS stimulation of BLaER1 cells lacking SARM1, there was a significant increase of TNF secretion compared to WT cells (Figure 1C), while CCL5 secretion was unaffected by absence of SARM1 (Figure 1D). In addition, mRNA levels of TNF were increased in SARM1 deficient cells at 6 h of LPS stimulation (Figure 1E). Consistent with the results from primary monocytes (Figure S1), there was no difference in CL075-stimulated TNF nor CCL5 in cells deficient in SARM1 compared to WT cells (Figures 1F and 1G). Together, the results thus far clearly show that SARM1 negatively regulates TLR4-dependent TNF secretion in human monocytes, most likely due to negative regulation of TRIF by SARM1.[7]

As well as TNF, we also observed enhanced IL-1β secretion after LPS treatment, but only at 12 h post-stimulation and not at earlier or later time points (Figure 1H). This was not the case for CL075-stimulated IL-1β secretion (Figure 1I). Unlike the case for TNF, there was no significant difference between IL-1β mRNA expression in LPS-stimulated SARM1-KO and WT cells (Figure 1J), although the data trended toward enhanced IL-1β mRNA induction in SARM1-KO cells at the 6 h time point. Since the effect of absence of SARM1 was more pronounced and significant on IL-1β protein secretion compared to IL-1β mRNA induction, similar to the case in mouse BMDMs,[9] the data

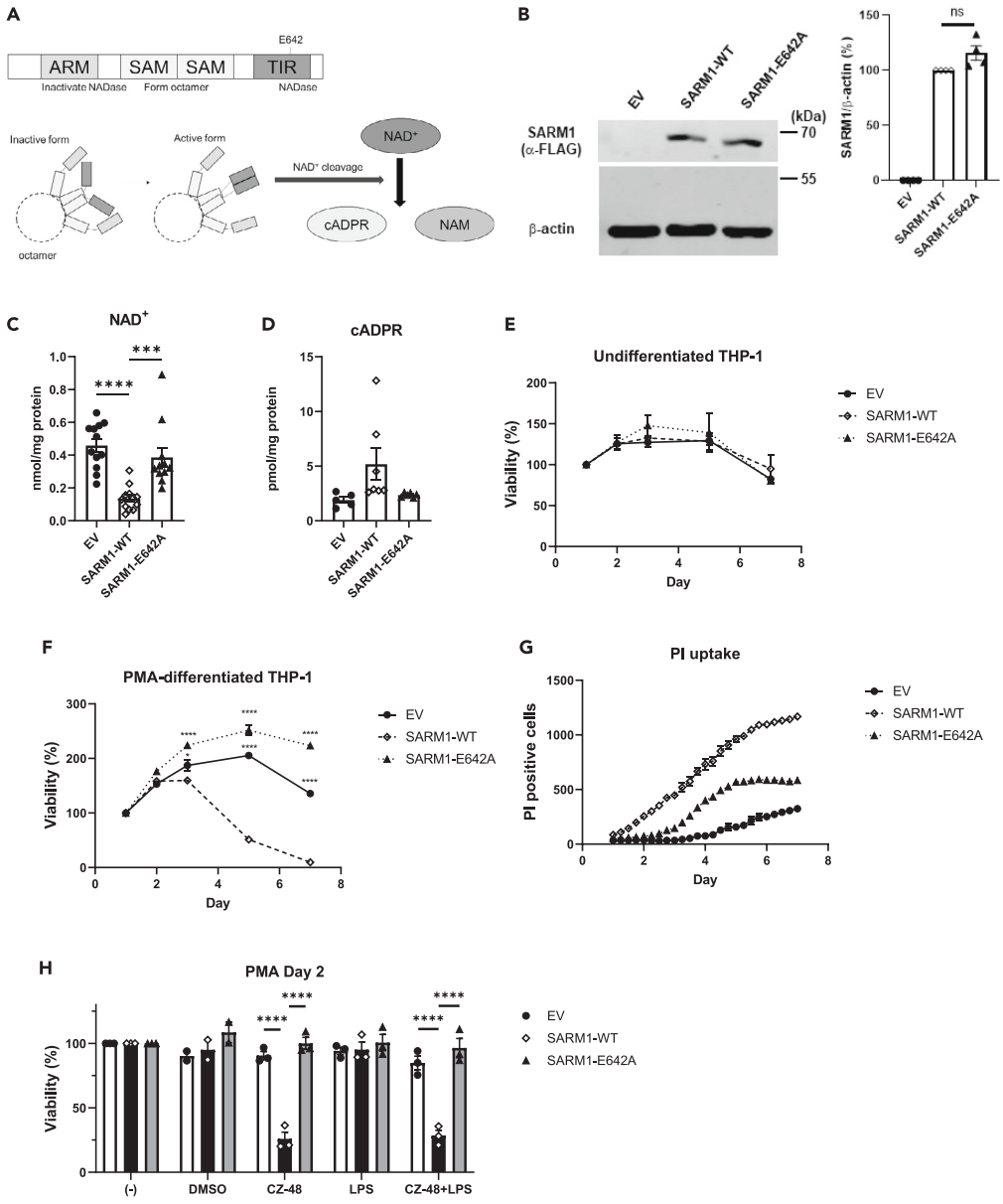

Figure 2. Characterisation of expression of enzymatically active and inactive SARM1 in monocytes

(A) Schematic of SARM1 domains and NADase enzymatic activity. The TIR domain has NADase activity and E642 is essential for this enzymatic activity. SARM1 forms an octamer, which is dependent on the SAM. To activate SARM1's NADase activity, dimerisation of TIR domains within the octamer is required, facilitated by a conformation change which distances the inhibitory ARM domain from the TIR domain. Active SARM1 cleaves NAD^+ and produces NAM and cADPR.

(B) Left panel, immunoblot showing stably expressing SARM1-FLAG protein in THP-1 cells stained with α-FLAG antibody. EV; empty vector control, SARM1-WT and SARM1-E642A. β-actin was used as a loading control. Representative of 4 experiments. Right panel, 4 independent experiments were performed and the FLAG-SARM1/β-actin ratio normalised to SARM1-WT was quantified by ImageJ. Data shown are mean ± SEM (bar chart) and each dot represents an independent experiment. Data were tested with an RM one-way ANOVA; Dunnett's multiple comparisons test.

(C) NAD^+ was assessed at day 5 of PMA-differentiated THP-1 cells. THP-1 cells seeded in 10 cm dishes were treated with PMA for 24 h followed by being cultured in PMA-free media. Data shown are mean ± SEM of 3 independent experiments, each performed in triplicate (3 dishes were prepared) or quadruplicate (4 dishes were prepared). Dots show the results of each dish. Data were tested with a one-way ANOVA; Tukey's multiple comparisons test, ***$p < 0.001$, ****$p < 0.0001$.

(D) cADPR were measured at day 3 of PMA-differentiated THP-1 cells. THP-1 cells seeded in 10 cm dishes were treated with PMA for 24 h followed by being cultured in PMA-free media. Data shown are mean ± SEM of 3 independent experiments, each performed in duplicate (2 dishes were prepared) or triplicate (3 dishes were prepared). Dots show the results of each dish.

Figure 2. *Continued*

(E) Undifferentiated THP-1 cells were cultured in PMA-free media and MTT assay performed at indicated time points. MTT conversion to formazan at day 1 is used as normalization, set as 100% viability. Data shown are mean ± SEM of 3 independent experiments, each performed in triplicate. Data were tested with a two-way ANOVA; Tukey's multiple comparisons test.

(F) THP-1 cells were treated with PMA for 24 h followed by being cultured in PMA-free media and MTT assay performed at indicated time points. MTT conversion to formazan at day 1 is used as normalization, set as 100% viability. Data shown are mean ± SEM of 3 independent experiments, each performed in triplicate. Data were tested with a two-way ANOVA; Tukey's multiple comparisons test, *$p < 0.05$, ****$p < 0.0001$. Significances are shown as comparison with SARM1-WT stably expressing cells.

(G) PI uptake of PMA-differentiated THP-1 cells was measured every 6 h from day 1 to day 7. Cells were treated with PMA at day 0 followed by replacing media with PMA-free, PI containing media at day 1, prior to starting the scanning of images. Data shown are mean ± SEM of 3 independent experiments, each performed in quadruplicate. (see STAR methods section for the detail). Data were tested with a two-way ANOVA; Tukey's multiple comparisons test, results are shown in Table S2. Also, see Videos S1, S2, and S3 show representative images used for calculation. (H) MTT conversion to formazan at day 2 of PMA-differentiated THP-1 cells. Cells were treated with PMA for 24 h followed by being cultured in PMA-free, containing indicated stimulants media for 24 h. Unstimulated cells are used as normalization, set as 100%. Data shown are mean ± SEM of 3 independent experiments and each dot represents an independent experiment. DMSO is used as a control for CZ-48 and it's also treated in cells shown as LPS. Data were tested with a two-way ANOVA; Tukey's multiple comparisons test, ****$p < 0.0001$. See also Table S2 and Videos S1, S2, and S3.

suggested that human SARM1 might negatively regulate IL-1β secretion after LPS stimulation via an effect on the NLRP3 inflammasome. Also, Gaidt et al.[35] reported that in human monocytes an alternative pathway of NLRP3 inflammasome activation without signal 2 exists, which can be activated by LPS stimulation alone, as seen in Figure 1H. IL-1β secretion in human monocytes in response to TLR8 stimulation utilizes an NLRP3-independent pathway,[36] which would be consistent with the lack of effect of SARM1 absence on CL075-stimulated IL-1β release in contrast to the effect on TLR4-stimulated IL-1β secretion. To further explore the role of human SARM1 in NLRP3 regulation, we treated BLaER1 monocytes primed by LPS with nigericin, in order to directly activate NLRP3.[35] We previously reported in mouse BMDMs that SARM1 regulates NLRP3 inflammasome outcomes by boosting pyroptotic cell death yet inhibiting IL-1β secretion.[9] Here we found that human SARM1 did have a slight inhibitory effect on IL-1β secretion but does not affect pyroptosis after nigericin treatment, since SARM1 deficiency caused no differences in pyroptosis determined by LDH release (Figure 1K) but did slightly but significantly increase IL-1β secretion (Figure 1L).

Expression of enzymatically active and inactive SARM1 in monocytes

Recent studies showed that the TIR domain of SARM1 in both mammals and lower organisms has NADase enzymatic activity.[14] Further, in some cases in lower organisms, the NADase activity has been shown to be required for innate immune functions of SARM1.[12] Although the SARM1 NADase activity is critical for mammalian SARM1-mediated neuronal cell death, to date no studies have investigated whether the innate immune functions of mammalian SARM1 require this activity. Having established that human SARM1 inhibits pro-inflammatory cytokine secretion (TNF, IL-1β) in monocytes, we wondered whether the NADase activity of SARM1 was required for this function. The NADase activity of SARM1 is abrogated by a single point mutation, E642A.[11] Thus, we initially attempted to establish SARM1-KO BLaER1 cells stably expressing WT SARM1 or E642A SARM1 using lentiviruses, but consistently failed in attempts to generate these cells. Instead, we took advantage of THP-1 cells, which are an established model for human innate immune studies, yet express undetectable levels of SARM1 protein (Figure 1B). This allowed us to constitute THP-1 cells with either normal SARM1 protein (SARM1-WT), or SARM1 protein where the NADase activity is abrogated (SARM1-E642A,[11]; Figure 2A), using a lentiviral expression system with puromycin selection, which enabled us to prepare THP-1 cells stably expressing either WT or E642A SARM1. Importantly, immunoblot showed that the WT and E642A protein were expressed at similar levels (Figure 2B), allowing us to directly compare the consequences of expression of NADase-active and -inactive SARM1 on innate immune responses. In order to confirm that SARM1-WT and SARM1-E642A functioned in this context as active and inactive NADases respectively, we measured NAD^+ and cADPR in THP-1 cells expressing these proteins, since cleavage of NAD^+ by endogenous SARM1 leads to generation of cADPR (Figure 2A, Essuman et al.[11]). In PMA-differentiated THP-1 cells, compared to empty vector (EV)-expressing cells we observed a significant reduction of NAD^+ in SARM1-WT cells but not in SARM1-E642A (Figure 2C). In addition, cADPR was increased in SARM1-WT cells compared to both EV-expressing cells and SARM1-E642A cells (Figure 2D). The data show that SARM1, but not SARM1-E642A is an active NADase generating cADPR in PMA-treated THP-1 cells.

Activation of SARM1 NADase reduces monocyte viability

While doing these assays, we noticed that, although expression of SARM1-WT or SARM1-E642A had no detrimental effect on cell viability in undifferentiated THP-1 cells over a period of 7 days (as measured by an MTT assay to monitor mitochondrial health, Figure 2E), when cells were treated with PMA, SARM1-WT cells showed a dramatic reduction of mitochondrial health from 3 days after PMA treatment, while the viability of SARM1-E642A cells did not reduce (Figure 2F). Consistent with that, an alternative assay of cell integrity, namely using an Incucyte live-cell imaging system to monitor PI uptake, showed that SARM1-WT expressed in PMA-stimulated THP-1 cells reduced membrane integrity over 7 days (Figure 2G, Table S2, Videos S1, S2, and S3). Compared to EV cells, SARM1-E642A expressing cells also showed a slight increase of PI uptake, but not to the degree seen for the SARM1-WT cells. These results strongly suggest that the NADase activity of SARM1-WT, is active in PMA-differentiated THP-1 cells, leading to eventual cell death due to NAD^+ depletion.

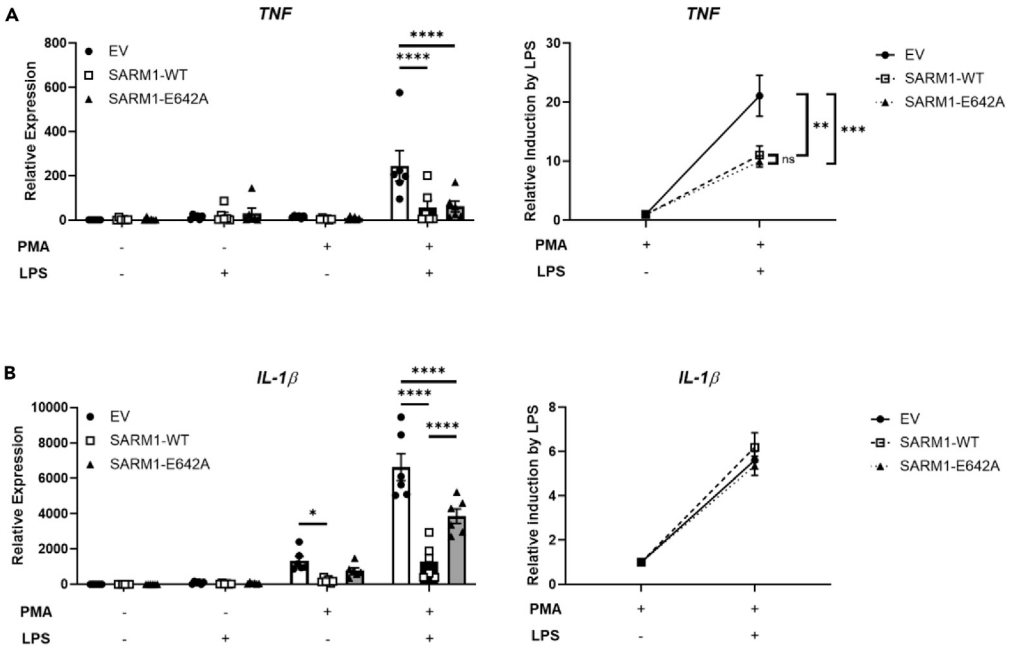

Figure 3. SARM1 inhibition of TLR4-stimulated TNF mRNA induction is NADase-independent

(A and B) Relative TNF (A) and IL-1β (B) mRNA were assessed by qRT-PCR, normalised to undifferentiated cells (left) or PMA-differentiated cells (right). Data shown are mean ± SEM of 6 independent experiments, each performed in triplicate or quadruplicate. Dots represent independent experiments (left). Data were tested with a two-way ANOVA; Tukey's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

Zhao et al.[32] reported that the small molecule activator of SARM1, CZ-48 (an NMN mimic) caused cell death in HEK293 cells stably expressing SARM1, due to the reduction of NAD^+ in the cell. Therefore we wondered if CZ-48 could also stimulate SARM1 NADase activity in THP-1 cells. Thus cells were treated with PMA for 24 h, followed by 24 h of LPS and/or CZ-48 in PMA-free media and MTT was measured. At this 48 h time point, PMA-treated SARM1-WT cells were fully viable compared to EV and SARM1-E642A cells (Figure 2F). However, CZ-48 treatment significantly reduced MTT conversion in SARM1-WT but not SARM1-E642A nor EV cells, both with or without LPS (Figure 2H). Together these results show that SARM1-WT is a viable NADase when expressed in THP-1 cells that can be activated both by extended PMA differentiation or by CZ-48 treatment. These observations allowed us to use this cell system to determine NADase-dependent and -independent effects of SARM1 on cytokine induction and inflammasome activation.

SARM1 inhibition of TLR4-stimulated TNF mRNA induction is NADase-independent

Previous data in the BLaER1 cells showed a clear role for SARM1 in LPS-stimulated TNF mRNA induction (Figure 1E) and a possible role for SARM1 in IL-1β mRNA induction (Figure 1J). To examine whether negative regulation of cytokine mRNA induction required SARM1 NADase activity, we examined TNF and IL-1β mRNA levels after LPS stimulation in both undifferentiated and PMA-differentiated THP-1 cells. In the absence of PMA differentiation, LPS did not cause induction of either TNF or IL-1β mRNA (Figures 3A and 3B). For TNF, LPS stimulation of PMA-treated THP-1 cells led to robust induction of mRNA, and this was potently, and equally, inhibited by SARM1-WT or SARM1-E642A expression (Figure 3A, left and right panels). This suggested that SARM1 does not utilise its NADase activity to negatively regulate TLR4-dependent TNF mRNA induction. For IL-1β mRNA, PMA alone actually caused potent induction of IL-1β mRNA, and this was inhibited by either SARM1-WT or SARM1-E642A expression (Figure 3B). When cells were treated with LPS, IL-1β mRNA was still inhibited by either SARM1-WT or SARM1-E642A expression (Figure 3B left panel), but not to a degree greater than PMA alone, when PMA stimulation was taken into account by normalising the data to PMA-differentiated cells (Figure 3B, right panel). These results show that both the potent inhibitory effect of SARM1 on TLR4-stimulated TNF mRNA induction and the more minor inhibitory effect of SARM1 on TLR4-stimulated IL-1β mRNA induction are independent of SARM1 NADase activity.

SARM1 regulates IL-1β secretion through NADase-dependent and -independent mechanisms

To examine regulation of IL-1β secretion by SARM1, THP-1 cells were differentiated by PMA treatment, primed with LPS and then stimulated with nigericin, leading to potent release of IL-1β (Figure 4A). Compared to cells expressing empty vector, there was a significant inhibition of IL-1β release in cells expressing SARM1-WT. This result in this gain-of-function SARM1 expressing system thus correlated with the enhanced IL-1β seen in the previous loss-of-function system in BLaER1 cells (Figure 1L), as did the fact that SARM1 expression had no effect on

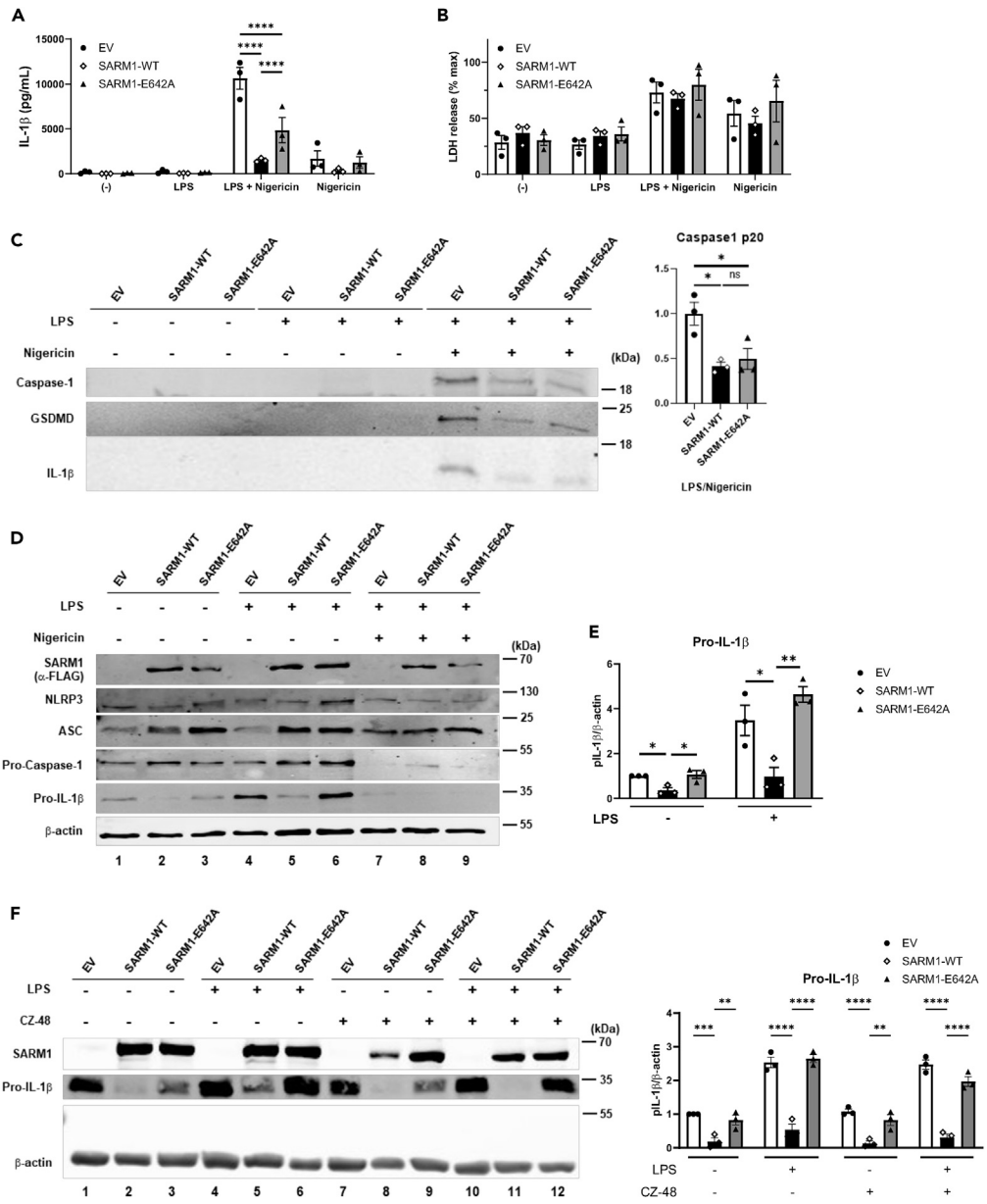

Figure 4. SARM1 regulates IL-1β secretion through NADase-dependent and -independent mechanisms

(A and B) PMA differentiated THP-1 cells were primed with LPS for 4 h, followed by nigericin stimulation for 2 h. Supernatants were collected to measure IL-1β protein secretion by ELISA (A) and LDH release (B). Data shown are mean ± SEM of 3 independent experiments, each performed in triplicate. Data were tested with a two-way ANOVA; Tukey's multiple comparisons test, ****$p < 0.0001$.

(C) Left panel, PMA differentiated THP-1 cells were primed with LPS for 4 h, followed by nigericin stimulation for 2 h. Immunoblot of supernatants from PMA-differentiated THP-1 cells with or without stimulation to activate NLRP3 inflammasome. Cleaved caspase-1 (p20), cleaved GSDMD (p30) and cleaved IL-1β (p17) are shown. Representative of 3 independent experiments. Right panel, 3 independent experiments were performed and Caspase 1 p20 was quantified by using ImageJ. Data shown are mean ± SEM of 3 independent experiments, each dot represents an independent experiment. Data were tested with a one-way ANOVA: Šídák's multiple comparisons test, *$p < 0.05$.

(D) PMA differentiated THP-1 cells were primed with LPS for 4 h, followed by nigericin stimulation for 2 h. Immunoblots of cell lysates from PMA-differentiated THP-1 cells with or without stimulation to activate NLRP3 inflammasome. Representative of 3 independent experiments. β-actin was used as a loading control.

(E) Quantification of pro-IL-1β from (D). Pro-IL-1β/β-actin ratios were normalised to PMA-treated EV control cells without stimulation. Data shown are mean ± SEM of 3 independent experiments, each dot represents an independent experiment. Data were tested with a one-way ANOVA: Šídák's multiple comparisons test, *$p < 0.05$, **$p < 0.01$.

Figure 4. *Continued*

(F) Left panel, PMA differentiated THP-1 cells were stimulated with LPS and/or CZ-48 for 6 h. Immunoblots of pro-IL-1β and SARM1 (to show no effect by NADase in other proteins) from cell lysates of PMA-differentiated THP-1 stimulated with LPS and/or CZ-48. Representative of 3 independent experiments. β-actin was used as a loading control. Right panel, quantification of pro-IL-1β and significance is assessed as described in (E). $^{**}p< 0.01$, $^{***}p< 0.001$, $^{****}p< 0.0001$.

pyroptosis stimulated by LPS and nigericin (Figure 4B). Interestingly, IL-1β release was also significantly inhibited by SARM1-E642A, albeit to a lesser degree than SARM1-WT (Figure 4A), suggesting that both NADase-dependent and -independent mechanisms whereby human SARM1 inhibits IL-1β secretion exist. Thus we next examined the effect of SARM1 on the various steps required for monocytes to elicit IL-1β secretion.

SARM1 regulates the NLRP3 inflammasome by an NADase-independent mechanism

NLRP3 activation by nigericin following LPS priming leads to cleavage of pro-caspase-1 to the active caspase-1, and subsequent caspase-1 processing of pro-IL-1β into the mature secreted form. IL-1β secretion also requires caspase-1 cleavage of GSDMD protomers leading to GSDMD oligomerization and membrane pore formation.[37,38] We previously showed that mouse SARM1 interacts with the NLRP3 inflammasome directly leading to inhibition of caspase-1 activation, and that inhibition of IL-1β secretion was mediated by the TIR domain.[9] To examine if human SARM1 regulates the NLRP3 inflammasome in the same manner as mouse SARM1, we stimulated THP-1 cells with LPS and nigericin and assessed cleaved caspase 1 (p20), IL-1β (p17), and GSDMD (p30). Similar to our previous findings in the mouse system, SARM1-WT inhibited activation (i.e., cleavage) of caspase 1, and also as a consequence GSDMD cleavage and appearance of mature IL-1β (Figure 4C). Interestingly, SARM1-E642A was equally capable of inhibiting caspase-1, GSDMD and the appearance of mature IL-1β, suggesting that SARM1 inhibits the NLRP3 inflammasome in a NADase-independent manner, which is likely a more significant contribution to the overall inhibitory effect of SARM1 on IL-1β secretion than any potential NADase-independent effect on IL-1β mRNA (Figures 1J and 3B).

SARM1 regulates pro-IL-1β protein expression by an NADase-dependent mechanism

We next wondered what aspect of the pathway to IL-1β secretion was sensitive to the NADase activity of SARM1, since we found that IL-1β secretion was inhibited by SARM1 in both an NADase-independent and -dependent manner (Figure 4A). This was revealed to us upon assessing the protein expression of inflammasome components, including pro-IL-1β, in the SARM1-WT-versus SARM1-E642A-expressing cells. As expected NLRP3, ASC, and pro-Caspase 1 protein expression were all expressed similarly in EV, SARM1-WT and SARM1-E642A cells (Figure 4D). Surprisingly however, we found that pro-IL-1β expression was significantly reduced in SARM1-WT cells compared to EV or SARM1-E642A cells for both unstimulated and LPS-treated monocytes (Figures 4D and 4E). This shows that pro-IL-1β expression is regulated by SARM1 NADase enzymatic activity, and thus NAD^+ depletion impairs pro-IL-1β (but not NLRP3, ASC or pro-Caspase-1) expression.

Finally, we treated cells with CZ-48, to directly activate SARM1 NADase and assessed pro-IL-1β protein expression with or without LPS in PMA-differentiated THP-1 cells. As previously (Figure 4D), pro-IL-1β protein expression was reduced in SARM1-WT compared to SARM1-E642A cells and pro-IL-1β protein expression was further reduced by CZ-48 in LPS-stimulated SARM1-WT cells, but not affected at all by CZ-48 in SARM1-E642A cells (Figure 4F, compare lane 11 to lane 5). Taken together these results show that SARM1 NADase activity limits pro-IL-1β protein expression.

Together our results reveal both NADase-dependent and -independent mechanisms whereby SARM1 inhibits pro-inflammatory cytokine secretion in human monocytes.

DISCUSSION

Our study provides several insights regarding the regulation of pro-inflammatory cytokines by SARM1 in human monocytes. Specifically, we showed that [1] SARM1 negatively regulates TLR4-but not TLR8-dependent TNF secretion, via an NADase-independent mechanism; [2] SARM1 negatively regulates the NLRP3 inflammasome in an NADase-independent manner, thus contributing to suppressing of IL-1β secretion; [3] SARM1 negatively regulates PMA-induced IL-1β transcription in an NADase-independent manner; [4] SARM1 negatively regulates pro-IL-1β protein expression post-transcriptionally via its NADase activity. All our findings support that overall SARM1 plays a role as a negative regulator of inflammatory responses in human monocytes.

For mouse, SARM1 is known to be most highly expressed in neurons, but a lot less is known about SARM1 expression in human cells and tissues. Of the human cells tested by us here, we found, similar to mouse studies, that SARM1 protein was highly expressed in neuronal cells, but also robustly expressed in primary human blood monocytes and macrophages. In contrast to the case in mouse BMDMs,[34] we show that human SARM1 in monocytes regulates TNF secretion, suggesting a broader role for SARM1 in human compared to mouse myeloid cells. Our data, together with reports of immune regulation by mouse SARM1 outside of neurons,[10,39] demonstrate the importance of examining the immune regulatory role of SARM1 in various organs beyond the brain and neurons, especially given the ongoing development of SARM1 inhibitors to protect against neurodegeneration.[12,22]

SARM1 is unique in the mammalian TIR protein family as to date it is the only TIR protein shown to have NADase activity, even though the majority of TIR proteins tested in lower organisms have been shown to have this activity. Most if not all mammalian TIR proteins have functions in innate immunity. Since this study and our previous work has shown that mammalian SARM1 has an immune-regulatory role it was important to determine the potential contribution of the NADase activity in this role. Interestingly, we discovered both NADase-dependent and -independent mechanisms for immune regulation by SARM1. The ability of SARM1 to inhibit TLR4-dependent TNF transcription did not require the

enzyme activity of SARM1 and likely is mediated via TIR homotypic interactions with TRIF, as was previously shown by us.[7] Also, SARM1-dependent suppression of the human NLRP3 pathway to IL-1β secretion was also NADase-independent and thus likely follows a similar mechanism whereby mouse SARM1 inhibits NLRP3 via TIR-dependent molecular interactions with the NLRP3 inflammasome complex.[9] Thus, the NADase activity may not be required to interact with those proteins. Interestingly, SARM1 also inhibited PMA-stimulated IL-1β transcription in an NADase-independent manner, and future studies will be required to understand the mechanism and whether it also involves SARM1 TIR domain interactions.

Unlike other mammalian TIR proteins involved in innate immunity, SARM1 has retained the ancient TIR NADase activity seen in TIR domain proteins of lower organisms, and interestingly this activity also contributes to immune regulation by human SARM1 in monocytes. Thus despite only a minor effect of SARM1 on LPS-stimulated IL-1β transcription, LPS priming followed by NLRP3 activation by nigericin led to IL-1β secretion that was partially inhibited by SARM1-E642A but more so by SARM1-WT. This revealed a role for SARM1 NADase activity in suppression of IL-1β secretion, distinct from the NADase-independent inhibition of NLRP3 activity. Analysis of the protein expression of inflammasome components showed no effect of SARM1 on NLRP3, ASC or pro-caspase-1, however surprisingly pro-IL-1β protein expression was impaired when the SARM1 NADase was active, even though pro-IL-1β transcription was normal. Therefore this result reveals a further point of control for IL-1β secretion not previously appreciated, in that the stability or translation of pro-IL-1β is sensitive to SARM1-mediated NAD^+ depletion. This result was corroborated by the fact that activation of SARM1 enzyme activity by CZ-48 treatment further reduced pro-IL-1β protein expression (since SARM1-WT expression alone depleted NAD^+ and impacted on cell integrity over time, but treatment of cells with CZ-48 reduced mitochondrial integrity in the presence of SARM1-WT but not SARM1-E642A we assume that SARM1-WT displays a basal level of NADase activity which is further enhanced by CZ-48 treatment). Consistent with this notion, basal NADase activity of SARM1 leading to cADPR production has been demonstrated in mouse neurons.[40]

Similar to our findings, Hancz et al. reported that heightened NADase activity in macrophages reduced IL-1β secretion.[41] In that study, translocation of the *Streptococcus pyogene*s NADase (SPN) into macrophages reduced inflammasome-dependent IL-1β release from *Streptococcus* infected macrophages, since IL-1β secretion in infected macrophages significantly increased when they infected cells with bacteria expressing NADase-dead SPN compared to normal SPN.[41] Similar to our studies, there was no effect of the NADase activity on IL-1β transcription, inflammasome component protein expression, caspase 1 activity or pyroptotic cell death, suggesting that the NADase inhibited IL-1β secretion independent of an effect on the NLRP3 inflammasome. On the other hand, they showed no effect on pro-IL-1β protein levels which is a clear difference from our findings. However in a follow-on study they did report that SLO induces the ubiquitination of pro-IL-1β and causes its degradation by autophagy.[42] Because SPN is tightly linked to SLO stability, this may be one of the potential mechanisms that NAD^+ levels regulate IL-1β specifically. Also, consistent with our findings, another study reported reduced intracellular NAD^+ levels in inflammatory cells results in reduced production of inflammatory cytokines including IL-1β.[43] Thus, taken together, there are several lines of evidence that NAD^+ is required for optimal IL-1β secretion.

Hence, although further studies are required to elucidate how exactly NADase activity regulates IL-1β, it is likely that when human myeloid cells respond to the presence of a pathogen, for example via detection of LPS, a maximal IL-1β response requires a certain level of intracellular NAD^+. In contrast, previous studies showed that NAD^+ is negatively correlated with IL-1β secretion in mouse BMDMs.[44] But consistent with our findings, Yang et al.[45] reported that elevated NAD^+ enhanced IL-1β release but not TNF in LPS-primed human monocytes. They also reported that phosphorylation of several proteins on the TLR4 signal pathway was inhibited by NAD^+ depletion by the NAMPT inhibitor FK866, which led to reduced cytokine transcription and production, and thus concluded that intracellular NAD^+ is essential for the inflammatory responses. In addition, Gerner et al.[46] reported FK866 effectively suppressed cytokine synthesis in lamina propria mononuclear cells isolated from human patients with IBD. Although we found no role for SARM1 NADase activity in regulating transcription, the results from Yang et al.[45] may also reflect a role for NAD^+ levels in optimal IL-1β protein expression which is independent of NLRP3 inflammasome-dependent IL-1β secretion.

Decline in whole organism or tissue NAD^+ is a known symptom of aging,[47] as is increased chronic low-grade inflammation (so called 'inflammaging').[48] These phenomena have been thought to be independent events, but recent studies suggest that they are closely related.[13] Interestingly, the NADase CD38 was shown to promote NAD^+ decline during aging in murine macrophages,[49,50] however, those studies didn't consider whether SARM1 is activated during the aging process. Thus it will be interesting to assess the role of mammalian SARM1 in ageing-induced NAD^+ depletion and ageing-induced inflammation. Further roles for NAD^+ cleavage by SARM1 in monocytes are also likely, and thus not limited to axon degeneration.[9,10,51,52] Ours and other studies showed that CZ-48-activated overexpressed SARM1 in non-neuronal cells led to cell death.[32] Also, cADPR modulates Ca^{2+}[14] that affects various cellular processes, and SARM1-dependent NAD^+ metabolism can lead to cADPR production. Thus future studies should further explore non-neuronal roles for SARM1 in cell physiology and immune and inflammatory responses.

In conclusion, our study shows that human SARM1 regulate pro-inflammatory cytokines expression in both NADase-dependent and -independent mechanisms in human monocytes. Our findings provide new insights of SARM1 as NADase enzyme play an important role beyond the role in neurons for the first time.

Limitations of the study

We did not consider any potential sex differences in SARM1 expression in primary human cells. It was difficult to completely validate the results obtained in the cell lines in primary human cells due to achieving only partial knock-down of SARM1 expression in primary human monocytes. Also we failed to rescue expression of SARM1 in SARM1-KO BLaER1 cells by using the lentivirus system which successfully established

SARM1-WT or SARM1-E642A expressing THP-1 cells. Thus, our findings rely on different cell models for gain-of-function and loss-of-function approaches. Also further studies are required to determine how exactly increased NADase activity limits pro-IL-1β protein.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

Conceptualization, R.S. and A.G.B.; methodology, R.S., K.A.S. and G.M.D.; investigation, R.S.; writing-original draft, R.S. and A.G.B.; supervisions, G.P.D. and A.G.B.; funding acquisition, R.S. and A.G.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
SARM1	ProSci	Cat# 3295, RRID:AB_735483
SARM1	Cell Signaling Technology	Cat# 13022, RRID:AB_2798090
FLAG	Sigma-Aldrich	Cat# F1804, RRID:AB_262044
NLRP3	AdipoGen	Cat# AG-20B-0014, RRID:AB_2490202
ASC	Santa Cruz Biotechnology	Cat# sc-22514-R, RRID:AB_2174874
Caspase 1	AdipoGen	Cat# AG-20B-0048, RRID:AB_2490257
Caspase 1 p20	Cell Signaling Technology	Cat# 4199, RRID:AB_1903916
IL-1β	R&D Systems	Cat# AF-201-NA, RRID:AB_354387
GSDMD	Sigma-Aldrich	Cat# HPA044487, RRID:AB_2678957
β-actin	Sigma-Aldrich	Cat# A5316, RRID:AB_476743
IRDye 680RD Goat anti-Mouse IgG	Li-cor	Cat# 926–68070, RRID:AB_10956588
IRDye 800CW Goat anti-Rabbit IgG	Li-cor	Cat# 926–32211, RRID:AB_621843
IRDye 800CW Donkey anti-Goat IgG	Li-cor	Cat# 926–32214, RRID:AB_621846
Biological samples		
Buffy coats of blood packs	Irish Blood Transfusion Service	N/A
Chemicals, peptides, and recombinant proteins		
β-Estradiol	Sigma-Aldrich	Cat# E8875
Human recombinant IL-3	Peprotech	Cat# 200-03
Human Macrophage Colony Stimulating Factor (M-CSF)	Peprotech	Cat# 300-25
Phorbol 12-myristate 13-acetate (PMA)	Invivogen	Cat# tlrl-pma
LPS	Enzo Life Sciences	Cat# ALX-581-010-L002
CL075	Invivogen	Cat# tlrl-c75
Nigericin	Invivogen	Cat# tlrl-nig
Sulfo-ara-F-NMN (CZ-48)	MedChemExpress	Cat# HY-129522
3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)	Sigma-Aldrich	Cat# M2128
Propidium Iodide (PI)	Invitrogen	Cat# P3566
Lipofectamine 2000	Invitrogen	Cat# 11668019
Polybrene	Sigma-Aldrich	Cat# H9268
Puromycin	Sigma-Aldrich	Cat# P8833-100MG
Critical commercial assays		
Human IL-1 beta/IL-1F2 DuoSet ELISA	R&D Systems	Cat# DY201
Human TNF-alpha DuoSet ELISA	R&D Systems	Cat# DY210
Human CCL5/RANTES DuoSet ELISA	R&D Systems	Cat# DY278
CytoTox 96 Non-Radioactive Cytotoxicity Assay (LDH assay)	Promega	Cat# G1780
Pierce BCA Protein Assay Kits	ThermoFisher	Cat# 23225
Neon Transfection System	Invitrogen	Cat# MPK5000S
Neon Transfection System 10 μL Kit	Invitrogen	Cat# MPK1096

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
BLaER1	Gift from Dr Thomas Graf, Center for Genomic Regulation, Barcelona, Spain	N/A
THP-1	ECACC	Cat# 88081201
HEK293T	ECACC	Cat# 12022001
SH-SY5Y	Sigma-Aldrich	Cat# 94030304
Oligonucleotides		
ON-TARGETplus Human SARM1 siRNA SAMRTpool	Dharmacon	Cat# L-008076-01
ON-TARGETplus Non-targeting Control pool	Dharmacon	Cat# D-001810-10
qPCR primers: see Table S1	This paper	N/A
Recombinant DNA		
pSpCas9(BB)-2A-Puro (PX459) V2.0	Gift from Feng Zhang[53]	Addgene plasmid # 62988
PX459-human SARM1 gRNA, target sequence: TGTCGCTTCTTCGCCATGTC	This paper	N/A
pdlNotInPkMCSR lentiviral expression vector	Previously described Carty et al.[9]	N/A
Human SARM1-WT-FLAG in pdlNotInPkMCSR vector	This paper	N/A
Human SARM1-E642A-FLAG in pdlNotInPkMCSR vector	This paper	N/A
pMD2.G (VSV-G)	Previously described Carty et al.[9]	N/A
pCMVR8.91 (Gag/Pol, Tat and Rev)	Previously described Carty et al.[9]	N/A
Software and algorithms		
GraphPad Prism 9	GraphPad	Version 9.4.1
Odyssey Software	Li-cor	ver. 3.0.16
ImageJ-Fiji	NIH	ver 1.53
BioRender	BioRender	BioRender.com

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Andrew G. Bowie (agbowie@tcd.i.e.).

Materials availability

All reagents generated in this study are available from the lead contact with a Materials transfer Agreement.

Data and code availability

- This paper does not report any original code.
- All data reported in this paper will be shared by the lead contact upon request.
- Any additional information required to reanalyze the data reported in this paper is available form the lead contact upon request.

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Primary human monocytes

Human peripheral blood mononuclear cells (PBMCs) from anonymous healthy donors were obtained by informed consent from buffy coats of blood packs from the Irish Blood Transfusion Service, using Lymphoprep (Stemcell) gradient centrifugation. $CD14^+$ Monocytes were isolated from PBMCs by positive selection using EasySep Human CD14 Positive Selection Kit II (Stemcell) following the manufacturer's protocol.

Cell lines

BLaER1 cells were a gift from Dr Thomas Graf, Center for Genomic Regulation, Barcelona, Spain. THP-1 (originally from a one year old male) and HEK293T cells were purchased from ECACC. SH-SY5Y cells were purchased from Sigma-Aldrich. HEK293T cells were grown in DMEM + Glutamax medium, supplemented with 10% FCS (v/v) and 10 μg/mL penicillin-streptomycin. BLaER1 and THP-1 cells were grown in RPMI + Glutamax medium, supplemented with 10% FCS (v/v) and 10 μg/mL penicillin-streptomycin (cRPMI). SH-SY5Y cells were grown in DMEM/F12 + Glutamax medium, supplemented with 10% FCS (v/v) and 10 μg/mL penicillin-streptomycin. All cells were kept at 37°C with 5% CO_2. BLaER1 cells were differentiated into monocytes by treatment with 100 nM β-Estradiol (Sigma-Aldrich), 10 ng/mL human recombinant IL-3 (Peprotech) and 10 ng/mL human macrophage colony stimulating factor (M-CSF, Peprotech) for 7 days in 96 well plates prior to stimulation.[35] For THP-1 differentiation, cells were treated with 100 nM of Phorbol 12-myristate 13-acetate (PMA, Invivogen) for 24 h prior to stimulation or replacing medium for further analysis. Cells were confirmed as mycoplasma-free.

Generation of SARM1 deficient BLaER1 cells

SARM1-deficient BLaER1 cells were generated by using pSpCas9(BB)-2A-Puro (PX459) V2.0 plasmid which was a gift from Feng Zhang (Addgene plasmid # 62988).[53] Guide RNA was designed to target exon 1 of *SARM1*, described in Figure S1. The gRNA was cloned into the plasmid following the Zhang lab protocol. The plasmid was transfected into BLaER1 cells using a Neon electroporator. Following puromycin selection for 2 days, cells were sorted into 96 well plates with one cell per well using FACSAria Fusion Sorter. Single cells were screened by T7E1 assay and NGS was used to confirm successful knock-out.

Generation of THP-1 cells stably expressing SARM1

SARM1 coding sequence was subcloned from cDNA derived from BLaER1 cells. To generate a NADase dead mutant E642A, site directed mutagenesis was performed by PCR to generate a point mutation. Both SARM1-WT or SARM1-E642A constructs with FLAG tag at 3′prime were cloned into pdlNotInPkMCSR lentiviral expression vector. The expression vector (SARM1-WT, SARM1-E642A or Empty vector control) was transfected into HEK293T cells along with VSVG and GAGPOL vectors to produce viruses. The resulting viruses were introduced into THP-1 cells by culturing for 48 h in virus produced serum-free medium. Then cells were replaced into cRPMI with 5 μg/mL puromycin, to select the cells. To confirm puromycin selection, non-transduced control THP-1 cells were cultured in parallel with puromycin-containing medium and all cells confirmed dead after 48 h. Following 72 h of selection, cells were subcultured into fresh cRPMI with puromycin and selected for another 3 days. Then cells were cultured in cRPMI.

METHOD DETAILS

Antibodies and reagents

The antibodies and reagents used for biochemical experiments were as follows. Primary antibodies: SARM1 (ProSci #3295, for immunoblots for primary monocytes; CST #D2M5I for all other immunoblots), FLAG (Sigma-Aldrich #F1804), NLRP3 (Adipogen #AG-20B-0014), ASC (Santa Cruz #sc-22514R), Caspase 1 (AdipoGen #AG-20B-0048-C100), Caspase 1 p20 (CST #D57A2), IL-1β (R&D #AF-201-NA), GSDMD (Sigma-Aldrich #HPA044487), β-actin (Sigma-Aldrich #A5316). Secondary Antibodies: IRDye anti-mouse IgG, anti-rabbit IgG and anti-goat IgG (LI-COR). All chemicals were purchased from Sigma-Aldrich unless specified.

siRNA knockdown

To knockdown SARM1 in primary monocytes, ON-TARGETplus Human SARM1 siRNA SMARTpool (containing 4 distinct siRNAs targeting SARM1) or a pool of four non-targeting Control siRNAs (Dharmacon) were used. Cells were transfected with siRNA using the Neon electroporator (Thermo) with 200 pmol of siRNA per 2 × 10^6 cells at 1400 V, 30 ms, 1 pulse setting. The experiments were done at 72 h post siRNA transfection unless otherwise specified in the figure legends.

Cell treatments with stimulants

Lipopolysaccharide (LPS) serotype EH(100)Ra (ENZO) was used at 100 ng/mL to stimulate TLR4. CL075 (InvivoGen) was used at 1 μg/mL to stimulate TLR8. CZ-48 (MedChemExpress) was used at 100 μM to activate SARM1 NADase enzymatic activity.

Enzyme-linked immunosorbent assay

Quantification of secreted cytokines and chemokines was performed using ELISA kits from R&D: Human IL-1 beta/IL-1F2 DuoSet ELISA (R&D, DY-201), Human TNF-alpha DuoSet ELISA (R&D, DY-210) and Human CCL5/RANTES DuoSet ELISA (R&D, DY-278). ELISA was performed following the manufacturer's protocol.

Immunoblotting

Cells were washed with PBS and then lysed in ice-cold lysis buffer (50 mM Tris-HCl, pH8.0, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and cOmplete mini Protease Inhibitor Cocktail (Roche)). Following incubation on ice for 30 min, samples were centrifuged for 20 min at 15,000 rpm

at 4°C. The supernatants (lysates) were transferred to a fresh tube, mixed with Laemmli sample buffer and boiled for 5 min at 99°C. Media supernatants were collected and centrifuged for 5 min at 1000 rpm at RT, then proteins are concentrated with Strata Clean Resin (Agilent) by rotating O/N at 4°C. On the next day, supernatants were removed, the beads were mixed with Laemmli sample buffer and boiled for 5 min at 99°C. SDS-PAGE was carried out in 10 or 15% agarose gel, and proteins were transferred to a nitrocellulose membrane using the semi-dry transfer system. Membranes were blocked using a 5% (w/v) solution of skimmed milk powder in PBS-Tween (0.1% Tween, v/v), then incubated with antibodies as indicated. Membranes were read on the Li-Cor Odyssey system. Images were adjusted and quantitated using ImageJ software.

RNA isolation and RT-qPCR

The High Pure RNA Isolation Kit (Roche) was used to isolate total RNA from cells by following the manufacture's protocol. cDNA synthesis was performed using M-MLV reverse transcriptase (Promega) with random hexamers following the manufacture's protocol. Quantitative evaluation of mRNA was performed by the ΔΔCT method using the QuantStudio 3 Real-Time PCR system (Invitrogen). Sequences of the oligonucleotides used are presented in Table S1. β-actin was used as a housekeeping gene for all assays.

Cell viability assay

Pyroptosis was assessed by LDH release by using CytoTox 96 Non-Radioactive Cytotoxicity Assay kit (Promega) following the manufacture's protocol. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich M2128) was dissolved in 1 mg/mL in PBS and 200 μL of MTT solution was added per cell culture well (96 well plates) at the indicated time point following removal of culture medium. After a 2 h incubation at 37°C, the solution was removed completely and 200 μL of DMSO was added per well and plates incubated at 37°C for 20 min in the dark. The plates were then read at 595 nm by a spectrophotometric plate reader. For suspension cells, a modified MTT assay was used.[54]

PI uptake

THP-1 cells were differentiated with PMA for 24 h and replaced with PI added PMA-free medium prior to measuring PI uptake. The cell plates with PI medium were set into Incucyte Live-Cell Analysis Systems (Sartorius) and scanned once immediately which was set as day 1. The following scans were made every 6 h after the first scan, until day 7 of PMA treatments. Cells were treated in triplicate and nine images per well were taken. Three independent experiments were performed and the mean of PI positive cells per well is shown as a result of PI positive cells.

NAD^+ measurement

5 million THP-1 cells were seeded in a 10 cm dish with PMA to be differentiated, and the media was replaced to PMA-free cRPMI the next day. On day 5 post-seeding, cells were scraped, washed once with PBS and then resuspended in 500 μL of PBS. 4% of cells were taken for BCA assay (Pierce BCA Protein Assay Kit, Sigma-Aldrich) and protein concentrations were determined following manufactures protocol. Cells were pelleted, resuspended in 555 μL of 0.5 M perchloric acid (PCA) and incubated on ice for 5 min. Samples were spun down, 500 μL of supernatants were recovered and neutralised with 88 μL of 2.5 M potassium hydroxide (KOH). Following a further spin, samples were transferred to new tubes and kept in −80°C until analysis. NAD^+ was assessed using standard HPLC on a C18 GeminiR 3 μm column (Phenomenex), using the isocratic method.[55] The mobile phase consisted of 220 mM potassium phosphate, 0.3 mM tetrabutylammonium and 1% (v/v) methanol, pH 6.9. The method was run for 30 min at a flow rate of 1 mL/min at 30°C. Chromatograms were analyzed using Empower Pro Software.

cADPR measurement

The enzymatic cycling assay was used to measure cADPR[56] with modifications, as follows: Briefly, 5 million THP-1 cells were seeded in 10 cm dish with PMA to be differentiated, medium was replaced to PMA-free cRPMI on the next day and cultured until day 3. Cells were washed by PBS and harvested, then resuspended in 500 μL of PBS. 4% of cells were taken for determining protein concentrations. Leftover cell pellets were resuspended in 555 μL of 0.5M PCA and incubated on ice for 5 min. Following centrifugation of samples, 500 μL of supernatants were recovered and neutralised with 116 μL of 2.5 M KOH. Following another centrifugation, 400 μL of samples were transferred to fresh tubes and adjusted to pH 8.0 by adding 400 μL of 10 mM sodium phosphate, pH 8.0. Then 0.2 U/mL phosphodiesterase and 0.5 mM $MgCl_2$ was added to each sample and samples were incubated overnight at 37°C with shaking to remove contaminating nucleotides. Phosphodiesterase was removed from the samples by filtration before running the cycling assay. By charcoal treatment, NAD^+ was removed from alcohol dehydrogenase (ADH) and diaphorase. Firstly, 50 μL of reagent containing 0.3 μg/mL ADP-ribosyl cyclase (ADPr-cyclase), 30 mM NAM, and 100 mM sodium phosphate, pH 8.0 was added to 100 μL sample and incubated RT for 15 min. Then 100 μL of cycling reagent; 100 μg/mL ADH, 2% ethanol, 20 μM resazurin, 10 μg/mL diaphorase, 10 μM flavin mononucleotide, 10 mM nicotinamide, 0.1 mg/mL BSA and 100 mM sodium phosphate, pH 8.0 was added. The cycling reaction was allowed to proceed for 1–16 h, and the increase in fluorescence was measured every hour using a SpectraMax^R fluorescence plate reader with excitation at 544 nm and

emission at 590 nm cADPR concentration in sample was calculated by cADPR standard curve generated on the same assay and the results are indicated by cADPR (pmol)/whole protein (mg).

QUANTIFICATION AND STATISTICAL ANALYSIS

All data were analyzed with GraphPad Prism 9. All experiments were repeated at least three times. Data are presented as mean values ±SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$. Specific statistical tests are described in detail in figure legends.