CD38 produces nicotinic acid adenosine dinucleotide phosphate in the lysosome

Cheng Fang, Ting Li, Ying Li, Guan Jie Xu, Qi Wen Deng, Ya Jie Chen, Yun Nan Hou, Hon Cheung Lee*, Yong Juan Zhao*

Laboratory of Cytophysiology, Key Laboratory of Chemical Genomics, Peking University Shenzhen Graduate School, Shenzhen, China, 518055

Running title: Lysosomal CD38 and NAADP biogenesis

*To whom correspondence should be addressed: leehc@pkusz.edu.cn; zhaoyj@pkusz.edu.cn

Keywords: nicotinic acid adenosine dinucleotide phosphate (NAADP); CD38; lysosome; endocytosis; nanobody

ABSTRACT

Nicotinic acid adenosine dinucleotide phosphate (NAADP) is a Ca^{2+}-mobilizing second messenger that regulates a wide range of biological activities. However, the mechanism of its biogenesis remains controversial. CD38 is the only enzyme known to catalyze NAADP synthesis from NADP and nicotinic acid. CD38-mediated catalysis requires an acidic pH, suggesting that NAADP may be produced in acidic endolysosomes, but this hypothesis is untested. In this study, using human cell lines, we specifically directed CD38 to the endolysosomal system and assessed cellular NAADP production. First, we found that nanobodies targeting various epitopes on the C-terminal domain of CD38 could bind to cell surface-localized CD38 and induce its endocytosis. We also found that CD38 internalization occurred via a clathrin-dependent pathway, delivered CD38 to the endolysosome, and elevated intracellular NAADP levels. We also created a CD38 variant for lysosome-specific expression, which not only withstood the degradative environment in the lysosome, but was also much more active than wildtype CD38 in elevating cellular NAADP levels. Supplementing CD38-expressing cells with nicotinic acid substantially increased cellular NAADP levels. These results demonstrate that endolysosomal CD38 can produce NAADP in human cells. They further suggest that CD38’s compartmentalization to the lysosome may allow for its regulation via substrate access, rather than enzyme activation, thereby providing an reliable mechanism for regulating cellular NAADP production.

Introduction

Nicotinic acid adenosine dinucleotide phosphate, NAADP was first discovered as a calcium mobilizer in sea urchin eggs in 1995 (1). Since then, a large body of evidence has shown that it is a universal second messenger, regulating intracellular Ca^{2+} concentrations by mobilizing acidic Ca^{2+} stores in a wide range of cells (review (2-4)). But until now, some aspects of NAADP-signaling still remain enigmatic, including its biosynthesis.

CD38 is an interesting protein that has attracted attention for decades, from its original use as a cell typing marker(5), its functioning as a signaling enzyme (6,7) and as a surface receptor (8), to being a therapeutic target for cancer immunosuppression (9) (review (10)). We first proposed that CD38 is an enzyme because of its sequence similarity with Aplysia ADP-ribosyl cyclase, the first enzyme known to catalyze the synthesis of the Ca^{2+}-messenger, cyclic ADP-ribose (cADPR) from NAD^{+} (6,7,11,12). Subsequent work establishes that CD38 not only
can cyclize NAD to cADPR, but also catalyze a separate base-exchange reaction, exchanging the nicotinamide group of NADP with nicotinic acid (NA) to produce NAADP (2,7). To date, CD38 and the base-exchange reaction remain the only known pathway for the biogenesis of NAADP.

The mechanism of how CD38 can catalyze multiple reactions has been well studied and understood, including the structural determinants underlying the acidic requirement of the base-exchange reaction (13). The mechanism of how CD38 synthesizes NAADP in cells, however, remains to be elucidated. CD38 is generally believed to be a type II transmembrane protein with its catalytic carboxyl (C)-domain facing outside (14). We have recently documented that type III CD38 of opposite membrane orientation also exists in cells (15,16). Since the base-exchange reaction requires acidic condition, type III CD38 with its catalytic C-domain facing the neutral cytosol may not be effective for NAADP synthesis. The major acidic compartment in cells is the endo-lysosome. It is possible that the surface type II CD38 is delivered to the endo-lysosomal compartment through endocytosis and is exposed to the acidic environment suitable for NAADP synthesis. A critical requirement for such a proposal is that CD38 can survive the highly degradative environment of the endo-lysosomes. In this work, we directly address this issue by constructing and directing CD38 into the endo-lysosomal compartment by two independent procedures. The results show that CD38 not only survives lysosomal degradation but is also fully active in making NAADP, which activity is substantially enhanced by the supplementation of NA. The results indicate that the type II lysosomal CD38 can be the enzyme catalyzing the endogenous production of NAADP and suggest that the compartmentation may allow NAADP synthesis to be regulated by substrate access instead of enzyme activation.

Results

Endocytosis of CD38 triggered by nanobodies (Nbs) – We have previously developed a series of Nbs targeting three separate epitopes on the C-domain of CD38 and have used them to produce immunotoxins that are highly effective in killing CD38\(^+\)-multiple myeloma cells (17). This suggests that the Nbs may trigger CD38 endocytosis efficiently by binding to the surface type II CD38. To determine if the Nb-treatment could be a specific mean for directing CD38 into the endo-lysosomal compartments, we incubated human LP-1 myeloma cells that expressed high levels of surface CD38 with Nb-1053 or Nb-551, two Nbs targeting different CD38 epitopes (17), and measured the extent of endocytosis of surface CD38. An unrelated Nb against GFP, NbGFP (18) was used as a negative control.

The endocytosis experiments were carried out in three steps as diagramed in Fig. 1a. The first step was incubation of the Nbs with the cells on ice to stop endocytosis and allow saturated binding of Nbs to the surface CD38. The incubation temperature was then raised to 37 °C in the presence of extra Nbs to commence endocytosis. At various times afterward, endocytosis was stopped on ice and all the extracellular Nbs that were not internalized were stripped by a glycine treatment. The quantification of the internalized Nbs was done by Western blots. As shown in the panels labeled Nb in Fig. 1b, increasing amounts of Nb-1053 (panels labeled Nb-1053) or Nb-551 (panels labeled Nb-551) were measured in the cells during the 15 min-period of endocytosis. That NbGFP was not internalized (panels labeled NbGFP) indicates that the endocytosis was CD38 specific.

To facilitate the quantification of the endocytosed Nbs, we labeled the Nbs with an amine-reactive green fluorescent dye, Alexa FluorTM 488 Succinimidyl Esters. That the labeling did not interfere with the specific binding of the Nbs to the surface CD38 was shown by the staining of the wildtype (CD38\(^+\)), but not CD38-knockout (CD38\(^-\)), LP-1 cells by the fluorescent Nbs (Fig. 1c).

The Nb-induced endocytosis of CD38 was a general occurrence not unique to LP-1 cells, and was observed in the HeLa and HEK293 cells as well. This is shown in Fig. 1d using HeLa cells transfected and expressing surface CD38.
Endocytosis was induced as described above, except using the fluorescent Nbs instead. After glycine-stripping, confocal imaging showed the internalized green signals in both cells treated with fluorescent Nb-1053 (Fig. 1d, white arrow) or Nb-551 (Suppl Fig. 1b), but not with fluorescent NbGFP (Suppl Fig. 1b). In the control without the warming step (Fig. 1d, Endocytosis 0 min, internal) to allow endocytosis, no internal signals were observed. The same Figure also shows the effectiveness of the glycine-stripping procedure to eliminate all surface-bound Nbs (Fig. 1d, Internal, green fluorescence). Cells were also stained with anti-CD38 (Fig. 1d, purple signals), and the internalized Nbs (green) showed substantial co-localization with CD38 (Merge, cyan). The results indicate that the Nb-induced endocytosis observed was specifically mediated by the binding of the Nb to the surface CD38 and that the internalized Nbs and CD38 were substantially associated with each other in the intracellular organelles. This validated the use of the internalized fluorescent Nb as a measure for the endocytosis of CD38.

In addition to HeLa cells, similar results were also obtained using HEK293 cells (Suppl. Fig. 2), confirming that the specific binding of the Nb to the surface CD38 is generally effective in inducing its endocytosis, irrespective of the cell type. All three cell types were used in this study. HeLa and HEK293 cells do not express CD38 and allow the study of various CD38 constructs described later without the interference of the endogenous CD38, while the LP-1 cell is used as a model for the naturally expressed CD38.

To quantify the endocytosis process in LP-1 cells, flow cytometry was used to obtain the Nb fluorescence distribution in the cell populations (Fig. 1e). Before warming to initiate endocytosis and without glycine-stripping, all cells had high Nb fluorescence (x-axis, Fig. 1e, bottom histogram) with a fluorescence value around $2.5 \times 10^4$ units at the peak of the cell histogram. After glycine-stripping, essentially all the bound Nbs were removed, with the corresponding large reduction in the fluorescence at the cell peak (Fig. 1e, light blue line, 0). After warming, internalization progressed and the fluorescence at the cell peak increased for the first 9 min and then stabilized (Fig. 1e, 3-15 min). The ratios of the fluorescence at the cell-peak value to that of the total (before stripping) represents the percentage of the internalized Nb at various time and were plotted in Fig. 1f. The slope of the first three points (0, 3, 6 minutes) of the plot was used to determine the internalization rate constants ($K_e$) as previously described (19). The $Ke$ values of Nb-1053 and Nb-551 were similar at around 0.007 min$^{-1}$, indicating about 0.7 % of the initial surface CD38 was internalized in a minute.

**Nanobody-induced endocytosis of CD38 is mainly via the clathrin-dependent pathway** – Pharmacological inhibition and RNA interference were then used to decipher if the Nb-induced internalization of CD38 was through the clathrin-mediated endocytosis (CME) or clathrin-independent endocytosis (CIE) (20). Inhibitors of CME, PAO and Pitstop 2 (21) at sufficient concentrations, substantially blocked the Nb-induced endocytosis in HeLa cells (Fig. 2a), while MβCD or Filipin (21), inhibitors of CIE, did not, indicating that the Nb-induced endocytosis was mainly going through the CME pathway.

This was further validated by RNA interference, using siRNA targeting clathrin heavy chain (CHC) or caveolin-1 (Cav1), a principal component of caveolae membranes involved in CIE (22). Western blots confirmed the knockdown efficiency (Fig. 2b). The percentages of CD38 internalized at various time points after treatment with Nb-1053 were shown in Fig. 2c. The data indicated that CHC knockdown impaired the endocytosis, while knockdown of Cav1 showed little effect.

If the Nb-induced endocytosis is indeed mediated by CME, the internalized Nbs should co-localize with clathrin (CHC). This is the case as shown in Fig. 2d. Merging of the fluorescence micrographs showed substantial overlap of the Nb and CHC fluorescence in a Hela cell after 30 min of induced endocytosis. The statistical analysis of the co-localization of the two signals for a number of cells showed significant increase after the Nb-1053 treatment (Fig. 2e). In contrast, the co-localization between Nb-1053 and Cav1...
significantly decreased (Fig. 2f-g). The data confirmed that the Nb-1053-induced endocytosis of CD38 was mediated mainly by the clathrin-dependent pathway.

The internalized CD38 is in the endolysosomes – To determine whether the internalized CD38 reached the endo-lysosomes, we co-stained the internalized CD38/Nb complex in HeLa cells with two marker proteins, Rab5 for early endosomes and LAMP1 for lysosomes. As the confocal images and statistical analyses shown in Fig. 3a-d, the co-localization of CD38 with both Rab5 and LAMP1 increased significantly after endocytosis induced by Nb-1053, indicating that the CD38/Nb complexes entered endo-lysosomes after internalization.

We further checked the influence of bafilomycin A1 (Baf-A1), a specific lysosomal inhibitor (23), on the Nb-induced endocytosis. In LP-1 cells pretreated with Baf-A1, the percentage of internalized Nb-1053, as determined by the fluorescence assay described above, increased more rapidly as compared with the control without pretreatment (Fig. 3e). Similar results were obtained using Western analysis to determine the internalized Nb-1053 (black bars, Fig. 3f). Blocking the lysosomal degradation thus inhibited the breakdown of the endocytosed Nbs, resulting in its accumulation. Notably and in contrast, the total amount of CD38 (open bars, Fig. 3f) stayed relatively unaffected during the period of the Baf-A1 treatment. That Baf-A1 did not cause the accumulation of CD38 during the indicated treatment period suggests that the protein is relatively stable against lysosomal degradation. Results presented later on will further prove this directly.

Endocytosed CD38 Increases the cellular NAADP levels – We proceeded to determine if the induced endocytosis resulted in elevation of cellular NAADP levels. LP-1 cells were treated with Nb-1053 and the NAADP levels were measured at various times afterward by the cycling assay we developed previously (24). As shown in Fig. 4a, in presence of NAD, the co-substrate for the base-exchange reaction, NAADP levels increased progressively, while the NbGFP treated cells did not show significant increase. The total amounts of CD38 stayed constant during all the treatments (Fig. 4b). The inset in Fig. 4a shows significant increase in cellular NAADP levels, albeit subdued, even without supplementing the cells with exogenous NA.

NA is vitamin B3 and is naturally present in vivo, although its concentration in the culture media (IMDM/FBS) may be low and thus requiring supplementation. That the presence of NA can greatly enhance the elevation cellular NAADP levels (compare Fig. 4a and inset) raises a novel possibility that the compartmentation of CD38 in the endo-lysosomes can allow the cellular NAADP levels to be regulated, not by activation of the enzymatic activity of CD38, but by access of the substrate, NA. This proposal will be discussed in more details below.

It has previously been shown that natural ligands such as NAD can also induce the internalization of CD38 (25,26). We confirmed that treatment with NAD did induce internalization of CD38 and that the internalized CD38 were co-localized with lysosomes by immunostaining, as shown in Suppl Fig. 3a. The treatment of NAD also induced progressive increase in the cellular NAADP levels, which were further enhanced in the presence of NA (Suppl Fig. 3b). It thus appears that the endocytosis of CD38 and elevation of NAADP is a general process, not limited only to that observed with the nanobody treatment.

Engineering a lysosome-targeting CD38, LysoCD38 – Results described above indicate that surface CD38 directed into the endo-lysosomal compartment by endocytosis not only can survive the degradative environment but is also active in producing cellular NAADP. To provide direct support, we constructed a lysosomal CD38 (LysoCD38) specifically targeted to express in the lysosomes. Fig. 5a diagrams the construct. It is a fusion protein containing an N-terminal signal peptide for endoplasmic reticulum (ER) trafficking and a C-terminal segment of LAMP1 (S381-I417) for lysosomal retention, and in between was spliced an EGFP tagged C-domain of CD38 (EGFP-sCD38). EGFP-sCD38 was constructed as described previously by replacing the N-terminal tail and transmembrane segment of CD38.
of the native CD38 with EGFP (Fig. 5a, EGFP-sCD38) (27). We have previously shown that EGFP-sCD38 is expressed in the cytosol and is not glycosylated (27). Also constructed was the full-length wildtype CD38 tagged with EGFP at the C-terminus (CD38-EGFP). These constructs were introduced to HEK293 cells by lentivirus infection, as described in the Methods and the expression of all four proteins was found to be very efficient (Fig. 5b, e).

Native CD38 is a transmembrane protein glycosylated in the ER and Golgi apparatus (27,28). Consistently, de-glycosylation of CD38-EGFP by EndoH resulted in reduction in size as shown in the Western blot in Fig. 5b (3rd-4th lanes). As a control, EGFP-sCD38 is soluble, made in the cytosol and is thus not glycosylated. EndoH treatment indeed did not affect its size (Fig. 5b, 5th-6th lanes). LysoCD38 has the same soluble EGFP-sCD38 sandwiched between the ER and lysosomal sorting signals. EndoH treatment resulted in size reduction (Fig. 5b, 1st-2nd lanes), indicating it was translated and glycosylated in the ER and Golgi apparatus like the wildtype CD38.

Immunostaining and confocal imaging of the HEK293 cells showed that the LysoCD38 co-localized with LAMP1 (Fig. 5c, first row). Correspondingly, the 2D scattered graph of LysoCD38 and LAMP1 fluorescence showed that the points fall mainly along a 45-degree line (Fig. 5d, first graph), indicating good co-localization between LysoCD38 and LAMP1. Consistently, the Pearson’s correlation obtained from 21 cells reached 0.7135 ± 0.0279, with unity indicating perfect correlation. These results indicate that LysoCD38 was expressed predominantly in lysosomes. In contrast, EGFP and EGFP-sCD38 were cytosolic, while CD38-EGFP was in the plasma membrane (Fig. 5c-d, 2nd-4th row). Their Pearson’s correlation values were all low at around 0.2.

The lysosomal localization of LysoCD38 was further confirmed by testing the changes of its EGFP fluorescence following lysosomal alkalization. The EGFP fluorescence is pH-sensitive and increases with alkalization. Ammonium chloride (NH₄Cl) is a lysosomotrophic agent that can effectively alkalinize the endo-lysosome compartments (29). As seen in the micrographs and the time courses of the digitized fluorescence of the cells (Fig. 5e-f), the EGFP fluorescence of the LysoCD38-expressing cells rapidly (in 5 sec) increased after NH₄Cl addition, but not those expressing the other constructs. That the EGFP fluorescence was responsive to the alkalinization of the lysosomal pH, indicated that the EGFP-sCD38 portion of LysoCD38 was facing the lumen of the lysosomes. In contrast, the EGFP fluorescence signals (Fig. 5f) of the other three cell lines were not altered, consistent with their EGFP-tags facing either the cytosol or the external medium and thus not affected by NH₄Cl.

The engineered LysoCD38 is enzymatically active and effectively elevates NAADP in cells – That the fluorescence of LysoCD38 could be readily detected indicates it is intact and stable inside the endo-lysosomes. To determine whether it is enzymatically active, lysates were prepared from the four cell lines expressing various constructs. The amounts of the various forms of CD38 expressed in HEK293 cells were measured by Western blots (Fig. 6a). The NADP produced by the reversed base-exchange activity was assayed using NAADP and nicotinamide as substrates as described in Methods. As shown in Fig. 6b, LysoCD38, CD38-EGFP and EGFP-sCD38 were all active, while EGFP, as a negative control, was not. After normalizing with the amount of the expressed CD38 (Fig. 6a), LysoCD38 showed similar activity as the wildtype CD38 (Fig. 6c, open bars). Also measured was the NAD produced by the reversed ADP-ribosyl cyclase activity of the lysates using cADPR and nicotinamide as substrates (Fig. 6c, black bars). Consistently, both LysoCD38 and wildtype CD38 had similar cyclase activities.

We next investigated if LysoCD38 could elevate the cellular NAADP levels. HEK293 cells expressing either the LysoCD38 or the wildtype CD38 were supplemented with NA at pH 5 to facilitate its entry into the cells. The time courses of subsequent elevation of cellular NAADP levels are shown in Fig. 6d. Cells expressing LysoCD38 showed progressive increase in cellular NAADP.
levels. Cells expressing wildtype CD38 did not show much change, which is consistent with it having a type II orientation with the catalytic domain facing outside and thus requires endocytosis to enter the endo-lysosomes for NAADP production (cf. Fig. 4).

Discussion
NAADP is the most potent \( \text{Ca}^{2+} \) messenger known to date and mediates a wide range of biological functions (30,31). How it is produced in cells, however, is not well understood. Despite sparse literatures suggesting other unknown enzymes might be involved (32,33), CD38 is still the only one fully characterized (7) and documented to synthesize NAADP in a variety of cells (34-36). We have shown that the base-exchange reaction catalyzed by CD38 to produce NAADP from NADP and NA requires an acidic pH (7). This is because of the electrostatic repulsion of the negatively charged NA (at neutral pH) and the acidic residues of CD38, Glu146 and Asp155, preventing the access of the substrate to the catalytic site (13). Since the major acidic compartment in cells is the endo-lysosomes, it has long been suggested that endocytosis can deliver CD38 into an environment suitable for NAADP production (2-4,7,25,35,37).

This study addresses the issue directly and uses two independent means to deliver CD38 into the endo-lysosomes. Firstly, Nbs raised specifically against different epitopes on the C-domain of CD38 (17,38) were used to induce endocytosis. Up to 7-8% of either endogenously (LP-1) or exogenously (HeLa and HEK293) expressed CD38 can be effectively delivered to the endo-lysosomes in 10 min. Secondly, we utilized the lysosomal sorting signal to direct EGFP-tagged CD38 to the lumen of lysosomes. The construct was efficiently expressed in the endo-lysosomes and its fluorescence was responsive to luminal pH changes.

CD38 delivered to the endo-lysosomes by either method was shown to be stable, enzymatically active and can elevate cellular NAADP contents, fulfilling the critical requirement for it to be part of the endogenous mechanism for NAADP synthesis. Although we used Nb to artificially activate endocytosis, the process is naturally occurring continuously in cells during membrane recycling and retrieval, as well as activated by natural ligands. Indeed, external NAD has been shown to induce internalization of CD38 (25) and natural ligands of CD38 have been identified, including CD31 (39). For cells normally express surface CD38, the endogenous endocytosis should result in the constant presence of endo-lysosomal CD38 for NAADP synthesis.

In this study, we emphasize the use of the CD38 nanobody as a tool. But it should be noted that CD38 antibody is a well documented physiological stimulus that can produce multiple biological effects, including the activation of T cells (40), suppression of B lymphopoiesis (41), activation of c-cbl in myeloid cells (42), and stimulation of insulin release in pancreatic islets (43). The results described in this study should be relevant for elucidating the mechanisms of these effects.

CD38 is a highly unusual protein. It catalyzes different reactions to produce cADPR and NAADP that target respectively the endoplasmic and the lysosomal \( \text{Ca}^{2+} \)-stores in cells. The cyclization reaction that produces cADPR occurs at neutral pH, while the base-exchange reaction for producing NAADP requires an acidic condition (7). More unusually, we have recently shown that CD38 naturally exists in two opposite membrane orientations. On the cell surface, it is expressed as a type II membrane protein with its catalytic C-domain facing outside. Intracellularly, it is expressed as a type III protein with its C-domain facing the cytosol (16). The C-domain of type III CD38 interacts specifically with CIB1, a cytosolic regulator protein, and results in modulation of the cellular cADPR levels (16). Because of its membrane topology, the catalytic domain of the Type II CD38, either expressed on the cell surface or delivered to the endo-lysosomes by endocytosis, is excluded from the cytosol and is thus unlikely to be regulated by cytosolic mechanisms. Controlling substrate access to the compartmentalized CD38 in the endo-lysosomes would be a novel mean to
regulate NAADP production, without the need to modulate its enzymatic activity. Consistent with this proposal, we showed in this study that supplementing the cells with NA indeed can enhance the NAADP production by the endolysosomal CD38, indicating the substrate was limiting. To facilitate its entry into the cells, we lowered the pH to protonate NA. Several natural transporters for NA have been described, including hOAT10 (44), bilirubin transporters (45), SMCT1 (46) and AE2 (47). Likewise, entry of NADP could be mediated by transporters like the annexin 43 hemi-channel, which has been shown to transport NAD and its permeability is regulated by phosphorylation (48). Physiological activation of these transporters could increase access of the substrates to the endo-lysosomes and elevate cellular NAADP levels. It remains to be determined whether this could be a novel way to regulate the messenger level in cells, not by activating the synthesizing enzyme but by increasing the substrate availability.

**Experimental procedures**

*Reagents and antibodies* – Phenylarsine oxide (PAO) were from Abcam. GAPDH antibody, PItsTop2, methyl-β-cyclodextrin (MβCD), filipin, bafilomycin a1 (Baf-A1), nicotinic acid and reagents for cycling assay were purchased from Sigma Aldrich. LAMP1 antibody (#9091) and Rab5 antibody (#3547) were from CST. Clathrin HC (CHC) antibody (sc-271253), caveolin-1 (Cav1) antibody (sc-894) were from Santa Cruz Biotechnology. Myc-tag mouse monoclonal antibody were from Genescript. Anti-CD38 antibody were home-made (16). HRP- or Alexa fluorophores-conjugated secondary antibodies were from Thermo Fisher Scientific.

*Cells* – HeLa, HEK293 and HEK293T cells were purchased from ATCC. LP-1 cell line was a gift from Annie An (Peking University, China). Cell lines were authenticated by genetic profiling with polymorphic short tandem repeat loci. The cells were maintained in DMEM (HeLa, HEK293, HEK293T and stable cell lines originated from these cells) or IMDM (LP-1 and CD38-KO/LP-1(16)), supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (Thermo Fisher Scientific), and cultured at 37 °C in a humidified atmosphere with 5% CO₂.

*Constructs and siRNAs* – The prokaryotic expression vectors of Myc-tagged Nbs and constructs encoding EGFP, EGFP-sCD38 and CD38-EGFP are referred to our previous reports (17,27). The LysoCD38 was constructed by fusing the signal peptide for ER trafficking, EGFP-sCD38 and the lysosome retention signal sequence (the nucleic acid and protein sequences are in Supplementary data) and the chimeric gene was then subcloned to pLenti-puro viral vector (Addgene, #39481) by LR ligation through pENTR1A-GFP-N2 (FR1) (Addgene, #19364). For expression of CD38 without tag, CD38 gene was cloned to pLenti-puro with the strategy described above.

siRNAs were synthesized in GenePharma. Sequences are 5’-UUCUCCGAACGUGUCACGUtt-3’ (for scramble), 5’-GCAAUGAGCUGUUGAGAAGAtt-3’ (for CHC) and 5’-CCUGAUUGAGAUUCAGUGCtt-3’ (for Cav1).

*Preparation and labeling of nanobodies* – Nanobodies, Nb-1053, Nb-551 and NbGFP, were expressed and purified as described previously (17). To label the Nbs, 200 μg of purified Nbs were incubated with Alexa FluorTM 488 (AF488) NHS ester (A20000, Thermo Fisher Scientific) at a molar ratio of 1: 4 in 0.2 M sodium bicarbonate buffer, pH 8.3, overnight at 4 °C. The reaction was stopped by 20 mM Tris and the unreacted probes were removed by filtration using Centricon filters (Millipore, #MA54455). The labeled AF488-Nbs were aliquoted and stored at -80 °C until use.

*Transfection and infection* – The siRNAs were transfected to HeLa cells using Lipofectamine RNAiMAX (Thermo Fisher Scientific). Lentiviral particles were prepared by transfecting HEK293T cells with pLenti-vectors, pMD2.G and psPAX2 with Lipofectamine 2000 (Thermo Fisher Scientific) and cell infection was performed as described previously (27) followed by puromycin selection.

*Internalization assays and treatments with inhibitors* – LP-1 cells were incubated with
AF488-conjugated Nbs (50 nM) for 40 min at 4 °C, then shifted to 37 °C to initiate internalization. A portion of cells were removed at different time points and the surface-bound Nb was stripped with the glycine-stripping buffer (200 mM glycine, 150 mM NaCl, pH 3.0) at 4 °C followed by Western blots or flow cytometry (CytoFLEX, Beckman Coulter). HeLa or HEK293 cells expressing CD38 were pre-treated with different inhibitors of endocytosis in HBSS (Thermo Fisher Scientific) for 3.5 h followed by the half-an-hour internalization step triggered by AF488-conjugated Nb-1053 (1053-AF488) at normal growth conditions. The cells were collected after detachment by treating with 0.05% Trypsin-EDTA (Thermo Fisher Scientific) at 37 °C for 4 min and subjected to flow cytometry to quantify the internalized Nb after the glycine-stripping step as described above. To disrupt lysosomal degradation, the cells were pre-treated with 1 μM bafilomycin A1 (BafA1) for 4 h at normal conditions if not specialized in the text.

**Immunofluorescence microscopy** – HeLa cells stably expressing CD38 were seeded onto coverslips coated with 0.001% poly-L-lysine in 24-well plates 24 h before experiments. The cells were incubated with Nb-1053 at 4 °C for 40 min, and then shifted to 37 °C for a 30 min-internalization step. Then the cells were washed with pre-cold PBS once or 3 times with glycine-stripping buffer and then fixed with the indicated fixation buffer according to the manufacturers’ instructions of the antibodies used. Immunostaining was done with indicated antibodies and images were acquired using a LSM510 confocal microscope (Zeiss).

For the realtime recording of the EGFP fluorescence, HEK293 cells were attached to poly-L-lysine pre-treated coverslips and imaged using an inverted microscope (TI-E, Nikon, Japan) equipped with a 40x oil-immersion objective (S Fluor 1.3 NA, Nikon). The fluorescence from individual cells was digitized and recorded continuously for around 3 min using the software NIS-Elements.

**Measurement of intracellular NAADP levels** – It was done as described previously (24,49). Briefly, cells were lysed with 300 μl of 0.6 M perchloric acid. The precipitated proteins were removed and re-dissolved in 1 M NaOH and quantified with the Bradford method. The nucleotide extracts were subject to a cycling assay after neutralization with a Tri-n-octylamine/chloroform mixture. The amounts of NAADP measured were normalized to the total amount of protein determined with the Bradford assay.

**Measurement of base-exchange and ADP-ribosyl cyclase activities** – The cell lysates of HEK293 cells expressing different fusion proteins were washed with PBS using Centricons for several times to remove the endogenous NAD and NADP. A portion of the cleaned lysates was used for Western blot to quantify the amount of CD38 in an unit volume of the lysates. A half microgram portion of the cleaned lysate was incubated with 20 mM nicotinamide and 100 μM NAADP (for the reversed base-exchange activity) or cADPR (for the reversed cyclase activity) for various periods to produce NADP or NAD, respectively, which was detected by the cycling assay as described previously (49). The activities were presented as nM NAD(P)/min/unit CD38.

**Quantification and statistical analysis** – Quantification of the Western blots was performed with ImageJ software. Imaris Bitplane software was used to do the colocalization analysis of the confocal signals. NIS-Elements software was used in quantification of the fluorescence in live-cell imaging. Statistical analysis was done with the GraphPad Prism software and the significance of differences was analyzed by the unpaired Student’s t-test. Data shown in reported figures are mean ± SEM from at least three repeats.
Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions: H.C.L. and Y.J.Z. designed research; C.F., T.L., Y.L., G.J.X., Q.W.D., Y.J.C., Y.N.H. performed research; C.F., H.C.L., and Y.J.Z. analyzed data and wrote the paper.
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This study was supported by grants from National Science Foundation of China (#31571438 to Y.J.Z., and #31470815 to H.C.L.) and grants from Shenzhen Governments (JCYJ20160608091848749 to Y.J.Z and JCYJ20170412150609690 to H.C.L.).

The abbreviations used are: NAADP, nicotinic acid adenosine dinucleotide phosphate; cADPR, cyclic ADP-ribose; NA, nicotinic acid; Nbs, nanobodies; NbGFP, nanobody against GFP; CHC, clathrin heavy chain; Cav1, caveolin-1; CME, clathrin-mediated endocytosis; CIE, clathrin-independent endocytosis; Baf-A1, bafilomycin A1; ER, endoplasmic reticulum.
Lysosomal CD38 and NAADP biogenesis

Figure 1. Nanobodies triggered endocytosis of CD38. (a) Schematic diagram of the protocol for detecting and quantifying the internalized Nbs. (b) Internalization of Nbs detected by Western blots. LP-1 cells were incubated with Nbs followed by the internalization protocol (a) and Western blots probing CD38, Nbs (anti-Myc) and GAPDH. (c) Binding capacity and specificity of the fluorescently labeled Nbs. Wildtype and CD38-KO LP-1 cells were incubated with AF488-labeled Nb-1053, Nb-551 or NbGFP, as a negative control. The representative fluorescent images merged with the bright field were shown. (d) Visualization of internalized Nbs. HeLa cells stably expressing CD38, were incubated with Nb-1053 and subjected to the internalization protocol and immunostaining with anti-CD38. Confocal micrographs of CD38 (purple) and Nb-1053 (green) are shown. Co-localization of both is colored cyan. Total: without glycine-stripping; internal: with glycine-stripping. (e) LP-1 cells were incubated with AF488-labeled Nb-1053, followed by the internalization protocol and analyzed with flow cytometry. (f) The percentages of the internalized
Nbs were plotted versus the time of endocytosis and the internalization rate constants ($K_e$) were calculated as described in the text.

Figure 2. Nanobody-induced endocytosis of CD38 was mainly via the clathrin-dependent pathway. (a) CD38-overexpressing HeLa cells were incubated with DMSO or different concentrations of PAO (0.25, 0.5, 1, 2 μg/mL), Pitstop 2 (1.875, 3.75, 7.5, 15 μg/mL), MβCD (1.6, 8, 40, 200 μg/mL), or filipin (0.008, 0.04, 0.2, 1 μg/mL) in HBSS for 3.5 h at normal growth conditions. 1053-AF488 was added and incubated for an additional half an hour. The internalized 1053-AF488 was measured by flow cytometry as described in Fig. 1. (b-c) CD38-overexpressing HeLa cells were transiently transfected with siRNAs, siCHC, siCav1 or scramble siRNA (NC). The cells were harvested 72h post transfection and used in Western blots for protein quantification (b) or endocytosis assay triggered by 1053-AF488 (c). (d-g) CD38-overexpressing HeLa cells were incubated with Nb-1053. Before or after endocytosis for 30 min, the cells were fixed, co-immunostained with the antibodies against CD38 (d, f, green) and CHC (d, purple) or Cav1 (f, purple), and the colocalization of CD38 with CHC (e) or Cav1 (g) was analyzed with the Imaris software.
Figure 3. Internalized CD38 was detected in the endo-lysosomes. (a-d) HeLa cells overexpressing CD38 were co-immunostained with the antibodies against CD38 (a, b, green) and Rab5 (a, purple, early endosome marker) or LAMP1 (b, purple, lysosome marker), with or without stimulation by Nb-1053 for 30 min. Colocalization of CD38 with Rab5 (a) or LAMP1 (c) was imaged by confocal microscopy and quantified with the Imaris software (b, Rab5; d, LAMP1). (e-f) With 4-h pretreatment of 1 μM Baf-A1, DMSO as a negative control, LP-1 cells were incubated with 1053-AF488 (e) or Nb-1053 (f) at 4 °C, and shifted to 37 °C to allow endocytosis for different time periods (0, 5, 15, 30, 60 minutes). The amounts of the internalized Nbs were measured by flow cytometry (e) or Western blots (f).
Figure 4. Treatment with Nb-1053 elevated the intracellular NAADP levels. (a, b) LP-1 cells were treated with Nb-1053 or NbGFP for different time periods (0, 3, 12, 24 h) in the absence (Inset) or presence of 10 mM NA. The intracellular NAADP contents (a) and CD38 expression (b) were measured by the cycling assay or Western blots, respectively.
Figure 5. Construction and characterization of lysosome-targeted CD38, LysoCD38. (a) Schematic diagram of the constructs, including LysoCD38, EGFP-sCD38, CD38-EGFP and EGFP. SP, signal peptide; sCD38, C-terminal catalytic domain of CD38; LAMP1-TM-C, the transmembrane domain and C-terminal tail of LAMP1. (b) Lysate of HEK-293 cells stably expressing different recombinant proteins were analyzed by Western blotting with or without treatment of the endoglycosidase, EndoH. (c, d) The four cell lines were immunostained with antibody against LAMP1 and the resulting red signals, together with the EGFP green signals were captured under confocal microscope (c) and the co-localization between green and red signals was analyzed by Imaris software (d). (e) The EGFP fluorescence images of the four cell lines were recorded before and after the addition of 25 mM NH₄Cl using a Nikon microscope. (f) The fluorescence intensities of the observed cells were digitized and recorded in real time using the NIS-Elements software.
Figure 6. **LysoCD38 is active in making NAADP in vitro and in cells.** (a) The levels of three forms of CD38 were analyzed in the four HEK293 cell lines stably expressing EGFP, LysoCD38, CD38-EGFP and EGFP-sCD38 by anti-CD38, or anti-GAPDH as a loading control. (b) The time courses of the production of NADP by the reversed base-exchange activity in same lysates used in (a) were measured as described in Methods. (c) The NAD produced by the reversed cyclase activity of the lysates used in (a) were measured as described in Methods. The values were normalized to the amounts of the CD38 proteins expressed, presented as nM NAD(P)/min/unit CD38, and compared with that of the reversed base-exchange activities. (d) HEK293 cells expressing LysoCD38 or CD38-EGFP were treated with 10 mM NA at pH 5. The courses of the change in NAADP level were measured and presented as pmol NAADP/mg proteins.
CD38 produces nicotinic acid adenosine dinucleotide phosphate in the lysosome
Cheng Fang, Ting Li, Ying Li, Guan Jie Xu, Qi Wen Deng, Ya Jie Chen, Yun Nan Hou,
Hon Cheung Lee and Yong Juan Zhao

J. Biol. Chem. published online April 9, 2018

Access the most updated version of this article at doi: 10.1074/jbc.RA118.002113

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