

## ACAT1-associated Late Endosomes/Lysosomes Significantly Improve Impaired Intracellular Cholesterol Metabolism and the Survival of Niemann-Pick Type C Mice

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We previously demonstrated that macrophages exhibit endoplasmic reticulum fragmentation under cholesterol-rich conditions, which results in the generation of acyl-coenzyme A: cholesterol acyltransferase 1 (ACAT1)-associated late endosomes/lysosomes (ACAT1-LE). ACAT1-LE efficiently esterify free cholesterol *in loco*, even with abnormal egress of free cholesterol from late endosomes. Because impaired free cholesterol transport from late endosomes results in Niemann-Pick type C disease (NPC), the induction of ACAT1-LE is a potential therapeutic intervention for NPC. To examine the effects of ACAT1-LE induction on intracellular cholesterol metabolism, we incubated bone marrow-derived macrophages possessing NPC phenotype (*npc1*<sup>-/-</sup>) with methyl- $\beta$ -cyclodextrin-cholesterol complex (m $\beta$ CD-cho), a cholesterol donor. Immunofluorescence confocal microscopy revealed that m $\beta$ CD-cho treatment of *npc1*<sup>-/-</sup> macrophages resulted in significant colocalization of signals from ACAT1 and lysosome-associated membrane protein 2, a late endosome/lysosome marker. *npc1*<sup>-/-</sup> macrophages contained significant amounts of free cholesterol with negligible amounts of cholesteryl ester, while wild-type macrophages possessed the same amounts of both cholesterols. m $\beta$ CD-cho treatment also induced marked restoration of cholesterol esterification activity. m $\beta$ CD-cho administration in neonate *npc1*<sup>-/-</sup> mice improved survival. These results indicate that ACAT1-LE induction in *npc1*<sup>-/-</sup> mice corrects impaired intracellular cholesterol metabolism and that restoring cholesterol esterification improves prognosis of *npc1*<sup>-/-</sup>. These data suggest that ACAT1-LE induction is a potential alternative therapeutic strategy for NPC.

**Key words:** Niemann-Pick type C disease, acyl-coenzyme A: cholesterol acyltransferase 1, late endosomes, cholesterol, methyl- $\beta$ -cyclodextrin

### I. Introduction

Macrophages bearing numerous lipid droplets in human atherosclerotic plaques are called foamy macro-

phages. These lipid droplets primarily consist of cholesteryl ester and triglycerides originating from modified or native low-density lipoprotein (LDL). Peripheral blood monocyte-borne macrophages internalize modified LDL via various scavenger receptors [16] and take up native LDL via fluid-phase pinocytosis [2, 13]. Internalized modified or native LDL is transferred to late endosomes/lysosomes (LE/LS), in which the cholesteryl ester in these particles is hydrolyzed and released as unesterified cholesterol. The release

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of unesterified cholesterol is mediated by Niemann-Pick type C1/C2 protein (NPC1/2) [5, 28]. Free cholesterol released from LE/LS is transferred to the plasma membrane, while excess free cholesterol migrates to the endoplasmic reticulum (ER), where it is re-esterified by acyl-coenzyme A: cholesterol acyltransferase (ACAT), after which macrophages accumulate esterified cholesterol as lipid droplets in their cytoplasm, which results in the transformation to foamy macrophages [5]. To date, two ACAT isozymes, ACAT1 and ACAT2, have been identified [1, 25]. ACAT1, but not ACAT2, is highly expressed in macrophage-derived foam cells in atherosclerotic plaques, and the ACAT1 expression is significantly upregulated during monocyte differentiation into macrophages [21]. ACAT1 is an enzyme that resides in the tubular ER membrane in various cell types [26]. We previously demonstrated that cholesterol-loaded foamy macrophages produce numerous ACAT1-positive, ER-derived small vesicles, some of which are functionally associated with LE/LS [15, 26, 27]. A quantitative confocal analysis disclosed that ACAT1 is not associated with the LE/LS marker protein LAMP2 in normolipidemic human macrophages, whereas approximately 20% of ACAT1 colocalizes with LAMP2 and forms ACAT1-associated LE/LS (ACAT1-LE) in cholesterol-loaded foamy macrophages [15, 27]. In macrophages with these functional units, *i.e.*, ACAT1-LE, modified LDL-derived cholesterol is re-esterified, even when U18666A, a reagent that blocks the egress of free cholesterol from LE/LS, is present, which results in the treated cells having the NPC1/2-deficient phenotype [15].

Niemann-Pick type C disease (NPC) is an inherited, progressive neurodegenerative disorder primarily caused by mutation of the *NPC1* gene, which results in the massive accumulation of free cholesterol in LE/LS due to impaired cholesterol egress [7]. Elevated free cholesterol in LE/LS promotes acid sphingomyelinase inhibition, with the consequence being abnormal accumulation of sphingomyelin in NPC [9]. Methyl- $\beta$ -cyclodextrin (m $\beta$ CD), a reagent that forms soluble inclusion complexes with free cholesterol [22], has been used to extract free cholesterol from the plasma membrane as well as cholesterol-laden organelles of cells with the NPC1-null phenotype (*npc1<sup>-/-</sup>*) [6, 29]. The administration of m $\beta$ CD in *npc1<sup>-/-</sup>* mice eliminates sequestered cholesterol in LE/LS from affected organs, which markedly improves tissue damage and significantly extends survival [18]. The beneficial effects of m $\beta$ CD treatment in *npc1<sup>-/-</sup>* mice are believed to be a consequence of cholesterol removal and esterification, in addition to the suppression of inflammatory responses [18]. However, the details of the mechanisms underlying these m $\beta$ CD-mediated therapeutic effects have yet to be fully elucidated.

In the present study, we used the cholesterol donor m $\beta$ CD-cho, not m $\beta$ CD, to investigate whether the induction of ACAT1-LE improves intracellular cholesterol metabolism in *npc1<sup>-/-</sup>* macrophages. We also evaluated the effects of m $\beta$ CD-cho treatment in improving the survival of *npc1<sup>-/-</sup>* mice in comparison to that achieved with conven-

tional m $\beta$ CD treatment. In addition, we discussed possible mechanisms to explain how, with respect to ACAT1-LE formation, cholesterol donors prolong the lifespan of *npc1<sup>-/-</sup>* mice.

## II. Materials and Methods

### Materials

Dulbecco's modified Eagle medium (DMEM) with a low glucose, protease inhibitor mixture, sodium dodecyl sulfate (SDS), bovine serum albumin (BSA), saponin, dimethyl sulfoxide, ethylenediaminetetraacetic acid, m $\beta$ CD, paraformaldehyde, fetal bovine serum (FBS), granulocyte-macrophage colony-stimulating factor (GM-CSF) and free cholesterol was purchased from Sigma-Aldrich Japan (Osaka, Japan). Penicillin-streptomycin solution for the cell culture and hexane:2-propanol were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dithiothreitol (DTT), ECL reagent and the BCA Protein Assay Kit were obtained from Thermo Scientific (Rockford, IL, USA). Rabbit anti-mouse ACAT1 polyclonal antibodies were purchased from Cayman (Ann Arbor, MI, USA), rat anti-mouse lysosome-associated membrane protein 2 (LAMP2) and anti-mouse  $\beta$ -actin monoclonal antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and anti-rabbit IgG Alexa Fluor 488 and anti-rat IgG Alexa Fluor 546 were purchased from Molecular Probes (Eugene, OR, USA). Fluorescence mounting medium (Vectashield) was obtained from Vector Laboratories (Burlingame, CA, USA). [ $^3$ H]Cholesterol linoleate was obtained from American Radiolabeled Chemicals (St. Louis, MO, USA).

### LDL and acetylated LDL preparation

Human LDL ( $d=1.019-1.063$  g/ml) isolated from normolipidemic human plasma via sequential ultracentrifugation was modified to produce acetylated LDL, as previously described [21]. [ $^3$ H]Cholesteryl linoleate-labeled acetylated LDL with a specific radioactivity of  $5 \times 10^4$  cpm/ $\mu$ g protein was prepared as described elsewhere [10].

### Animal model

Mice with the *npc1<sup>-/-</sup>* phenotype were purchased from Jackson Laboratory (Bar Harbor, ME, USA). All animal experiments were carried out according to the guidelines of the Animal Experimental Ethics Committee of Kumamoto University.

### Cell culture

Bone marrow monocytic cells derived from *npc1<sup>-/-</sup>* or wild-type mice were suspended in basic medium (DMEM low glucose supplemented with 10% FBS, 0.1 mg/ml of streptomycin and 100 U/ml of penicillin G). The cells were seeded in 100-mm cell culture dishes ( $6 \times 10^6$  cells per dish), and adherent monocytic cells were incubated with 20 ng/ml of GM-CSF for seven days to induce differentiation into macrophages. The cell culture experiments described above

were carried out at 37°C in a humidified atmosphere with 5% CO<sub>2</sub> in air.

#### Immunocytochemistry, confocal microscopy and histochemistry

Macrophages cultured in 6-well tissue culture plates were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) on ice for 30 min and then were rinsed with Buffer A (phosphate-buffered saline (PBS) containing 0.5% BSA and 0.1% saponin) three times for five min each time. The cells were pretreated with 5% goat serum and incubated with primary antibodies for 60 min. After removing the primary antibodies using Buffer A, the cells were incubated with anti-rabbit Alexa Fluor 488 or anti-rat Alexa Fluor 546 for 60 min, excess secondary antibodies were removed by rinsing and coverslips were mounted on the slides. Digital images were scanned and recorded using the Olympus FV300 Confocal Fluorescence Microscope (Tokyo, Japan) at a resolution of 1024×1024 pixels/frame with an objective lens with a numerical aperture of 1.40 and an estimated optical thickness of less than 500 nm. The images were obtained by two independent researchers without information regarding the samples. In order to standardize the signal intensity, a representative image with apparent signal colocalization was obtained as a control (Fig. 1p, q, r), and other images were recorded under the same image

parameters (detector gain, amplification offset, amplification gain). For the quantitative analysis of the confocal images, at least 20 images were scanned at random. Colocalization coefficients for the ACAT1 and LAMP2 signals were calculated using the WinROOF image analysis software package, version 5.7 (Mitani Corp., Tokyo, Japan).

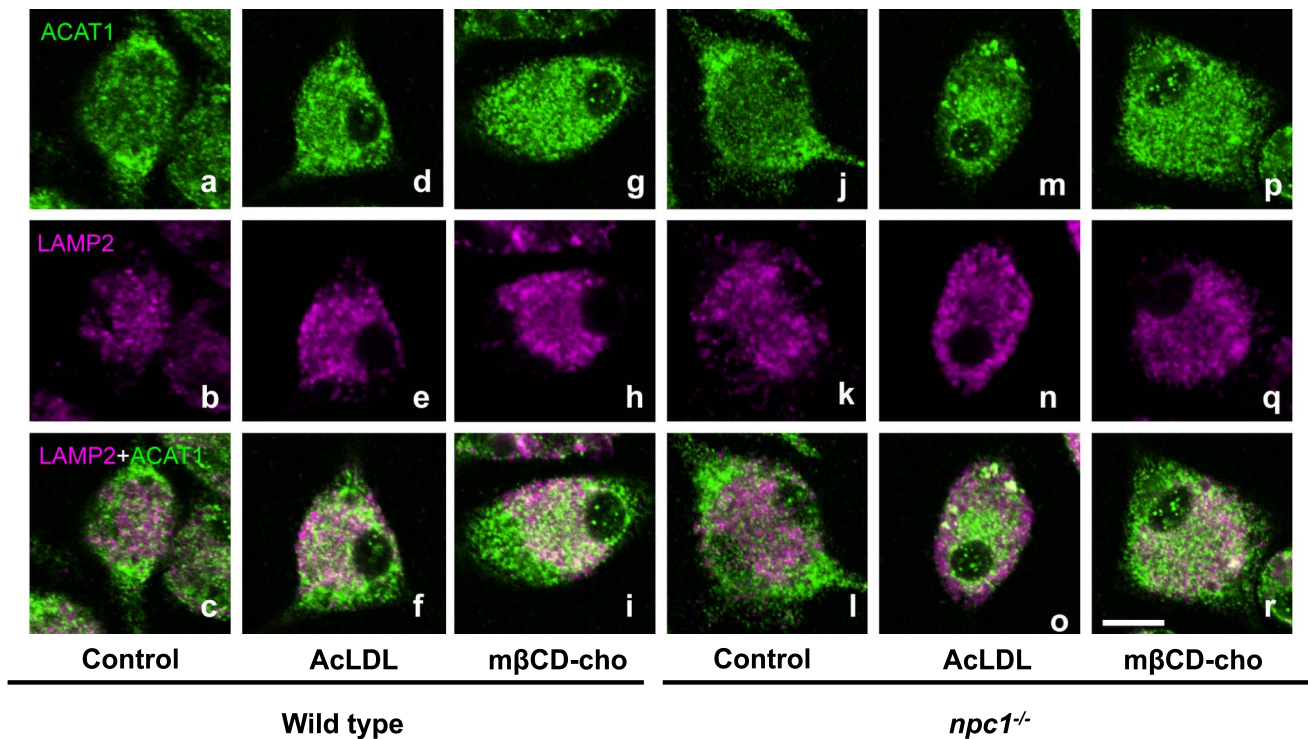
In order to detect cholesteryl ester as lipid droplets, after fixation, the cells were immersed in Oil Red O solution for 30 min at 37°C. After removing the excess Oil Red O dye by washing with 60% 2-propanol and running water, the cells were counterstained with hematoxylin and mounted on slides with coverslips.

#### Immunoblot analysis

Total cell lysates containing 0.1 M DTT and 10% SDS were incubated at 37°C for 60 min. The solubilized proteins were run on 10% SDS-polyacrylamide gel electrophoresis (PAGE) and subjected to immunoblotting, as previously described [4].

#### Quantitative analysis of intracellular cholesterol and cholesteryl ester

Macrophages incubated with 50 µg/ml of acetylated LDL or mβCD-cho (250 µM of mβCD containing 16 µM of cholesterol) for 48 hr or without acetylated LDL or mβCD-cho were rinsed with PBS three times and dried



**Fig. 1.** Colocalization of ACAT1 and LAMP2 in wild-type and *npc1*<sup>-/-</sup> murine macrophages following treatment with acetylated LDL (AcLDL) or mβCD-cho. Wild-type and *npc1*<sup>-/-</sup> murine macrophages treated for 48 hr with 50 µg/ml of acetylated LDL or mβCD-cho (250 µM of mβCD containing 16 µM of cholesterol) were fixed and immunostained as described in the Materials and Methods section. Representative immunofluorescent images of control and cholesterol-loaded macrophages. Green indicates ACAT1, magenta indicates LAMP2 and white indicates colocalization.

at room temperature. Cellular lipids were extracted using hexane:2-propanol (3:2), and the levels of total cholesterol and free cholesterol were determined using a cholesterol assay kit according to the manufacturer's instructions (Wako Chemical Industries, Tokyo, Japan). The amount of cholesteryl ester was calculated by subtracting the amount of free cholesterol from the amount of total cholesterol. The level of cellular protein dissolved in 0.1 N sodium hydroxide was determined using the BCA Protein Assay Kit, with BSA as the standard.

#### **Modified LDL-derived cholesterol re-esterification assay**

Macrophages cultured in 24-well tissue culture plates at a density of  $0.2 \times 10^6$  cells per well were incubated for 48 hr with one of three media: basic medium, medium with 50  $\mu\text{g/ml}$  of acetylated LDL or medium with m $\beta$ CD-cho (250  $\mu\text{M}$  of m $\beta$ CD containing 16  $\mu\text{M}$  of cholesterol). After washing the cells three times with PBS, they were incubated with basic medium containing 50  $\mu\text{g/ml}$  of [ $^3\text{H}$ ]cholesteryl linoleate-labeled acetylated LDL for 24 hr. The radio-labeled medium was then removed, and the total lipids in the cells were extracted with hexane:2-propanol (3:2) and subjected to silver nitrate-impregnated thin-layer chromatography, which separated cholesteryl oleate from cholesteryl linoleate, according to the procedure described previously [3]. The radioactivity of [ $^3\text{H}$ ]cholesteryl oleate was determined with a liquid scintillation counter.

#### **Effects of m $\beta$ CD administration on the survival of the *npc1*<sup>-/-</sup> mice**

In order to evaluate the effects of ACAT1-LE induction on the survival of the *npc1*<sup>-/-</sup> mice, day 7 neonates were injected with m $\beta$ CD-cho (250  $\mu\text{M}$  of m $\beta$ CD containing 16  $\mu\text{M}$  of cholesterol) dissolved in 1 ml of PBS. The animals were maintained under specific pathogen-free conditions until death.

#### **Statistics**

The data are presented as the mean $\pm$ SD. Statistical analyses of the results were performed using a one-way analysis of variance (ANOVA). A *p* value of less than 0.05 was defined as being statistically significant.

### **III. Results**

#### **ACAT1-LE formation by *npc1*<sup>-/-</sup> macrophages**

In the present study, we first used immunofluorescence confocal microscopy to confirm the association between ACAT1 and LE/LS in cholesterol-rich *npc1*<sup>-/-</sup> murine macrophages. As shown in Figure 1, the control macrophages exhibited ACAT1 and LAMP2 signals that were separately detected in both the wild-type and *npc1*<sup>-/-</sup> mice (Fig. 1a, b, c, j, k, l). In contrast, both signals were partially colocalized with each other after m $\beta$ CD-cho treatment, thus resulting in significant white signals (Fig. 1g, h, i, p, q, r). The quantitative analysis revealed that only 1–3% of the ACAT1 signals overlapped with the LAMP2 signals in the

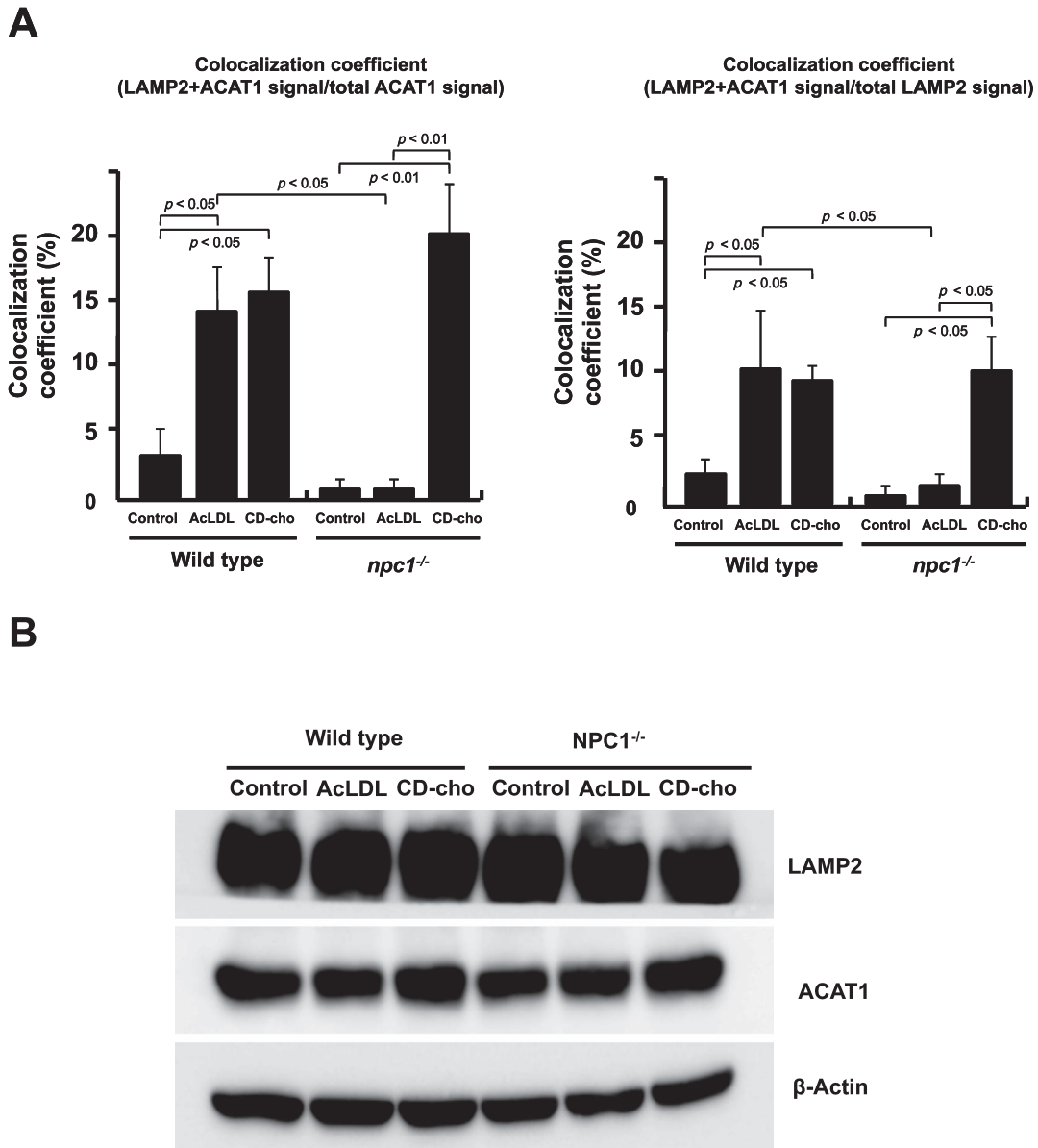
wild-type and *npc1*<sup>-/-</sup> macrophages grown in basic medium (control in Fig. 2A), whereas 10–20% of the respective signals overlapped in the wild-type and *npc1*<sup>-/-</sup> macrophages incubated with the cholesterol donor m $\beta$ CD-cho (CD-cho in Fig. 2A). As an interesting finding, the use of acetylated LDL, another cholesterol donor, revealed a significant association between ACAT1 and LAMP2 in the wild-type macrophages (Fig. 1d, e, f), whereas no changes were observed in the *npc1*<sup>-/-</sup> macrophages (Fig. 1m, n, o). The quantitative analysis also disclosed a significant association between both signals in the wild-type mice, but not in the *npc1*<sup>-/-</sup> mice (Fig. 2A). Furthermore, the expression levels of LAMP2 and ACAT1 did not change after cholesterol donor treatment compared with that noted in the controls (Fig. 2B), which suggests that ACAT1-LE formation in murine macrophages depends on ER fragmentation, similar to that observed in human macrophages [26]. Our data therefore indicate that *npc1*<sup>-/-</sup> macrophages produce ACAT1-LE and that m $\beta$ CD-cho, but not acetylated LDL, holds promise as a cholesterol donor for inducing ACAT1-LE in *npc1*<sup>-/-</sup> macrophages.

#### **Intracellular free cholesterol and cholesteryl ester levels**

In order to determine whether m $\beta$ CD-cho-induced ACAT1-LE corrects intracellular cholesterol esterification in *npc1*<sup>-/-</sup> macrophages, we analyzed the levels of cholesterol and cholesteryl ester after m $\beta$ CD-cho treatment. As shown in Figure 3A, m $\beta$ CD-cho treatment did not change the intracellular free cholesterol levels in the wild-type macrophages, whereas those observed in the *npc1*<sup>-/-</sup> macrophages decreased significantly, to levels equal to those detected in the wild-type macrophages. The levels of intracellular cholesterol esters, in contrast, increased following incubation with m $\beta$ CD-cho in both types of cells (Fig. 3B). Interestingly, the cholesteryl ester levels after m $\beta$ CD-cho treatment were significantly higher in the *npc1*<sup>-/-</sup> macrophages than in the wild-type macrophages. This result is obtained when both free cholesterol derived from m $\beta$ CD-cho and cholesterol accumulated in LE/LS are esterified by ACAT1-LE in *npc1*<sup>-/-</sup> macrophages. Histochemical staining with Oil Red O confirmed that the *npc1*<sup>-/-</sup> macrophages without neutral lipid accumulation transformed into foamy macrophages after m $\beta$ CD-cho treatment, as did the wild-type macrophages (Fig. 3C). These data thus indicate that m $\beta$ CD-cho induces ACAT1-LE to eliminate abnormal free cholesterol, resulting in cholesteryl ester formation in *npc1*<sup>-/-</sup> macrophages.

#### **Modified LDL-derived cholesterol re-esterification assay**

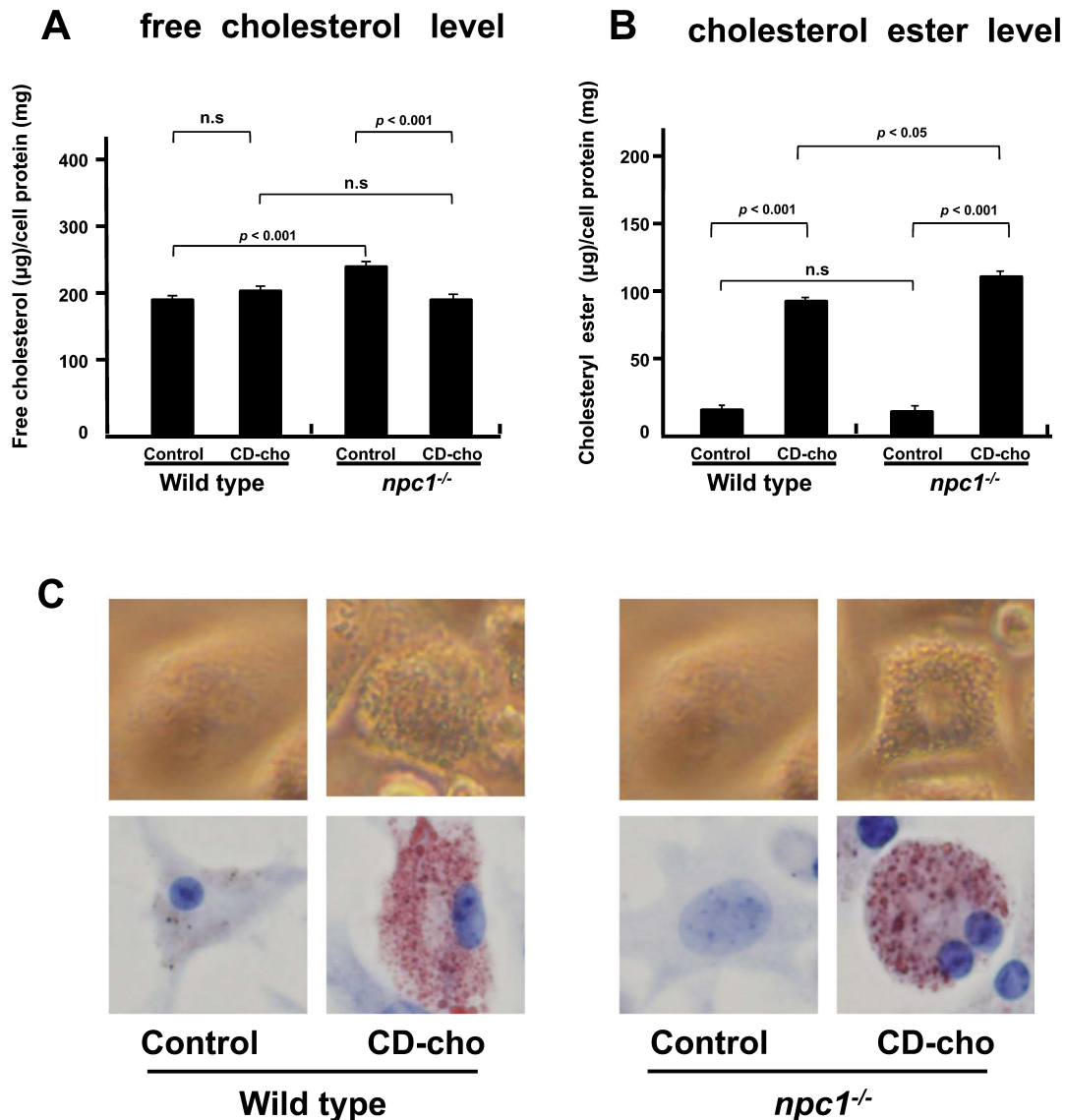
We next investigated the functional significance of ACAT1-LE formation in *npc1*<sup>-/-</sup> macrophages. Earlier studies have shown that treatment with the amphipathic amine U18666A induces functional deficiency in cells, which results in the accumulation of free cholesterol in LE/LS [7, 12, 14, 17]. The induction of ACAT1-LE in human macrophages facilitates the access of ACAT1 to free cholesterol trapped in LE/LS and restores cholesterol esterifi-



**Fig. 2.** Quantitative analysis of the association between ACAT1 and LAMP2 in wild-type and *npc1*<sup>-/-</sup> murine macrophages following treatment with AcLDL or m $\beta$ CD-cho. **(A)** Quantitative evaluation of the colocalization coefficient for the ACAT1 and LAMP2 signals. Immunofluorescent signals from all samples were scanned and analyzed as described in the Materials and Methods section. The results represent the findings of three separate experiments. **(B)** Immunoblot study of the expression levels of ACAT1 and LAMP2 proteins in the wild-type and *npc1*<sup>-/-</sup> murine macrophages following treatment with acetylated LDL or m $\beta$ CD-cho. The samples consisted of total cell lysate (20  $\mu$ g of protein), which was subjected to SDS-PAGE and an immunoblot analysis, as described in the Materials and Methods section.  $\beta$ -Actin was used as the internal control protein.

cation in human macrophages treated with U18666A [15]. In order to evaluate the restoration of cholesterol esterification in *npc1*<sup>-/-</sup> macrophages achieved via ACAT1-LE formation, we used a cholesterol re-esterification assay. Macrophages with or without the *npc1*<sup>-/-</sup> phenotype were incubated with [<sup>3</sup>H]cholesteryl linoleate-labeled acetylated LDL, and the amount of re-esterified [<sup>3</sup>H]cholesteryl oleate was determined. As shown in Figure 4, the re-esterification activity in the *npc1*<sup>-/-</sup> macrophages was quite suppressed

compared with that observed in the wild-type macrophages (approximately 5% of the control level). Following m $\beta$ CD-cho treatment, however, the *npc1*<sup>-/-</sup> macrophages exhibited a markedly higher esterification activity, and the relative esterification measured 80% of that observed in the wild-type macrophages. As for the experiments with U18666A, these results indicate that ACAT1-LE restores cholesterol esterification in *npc1*<sup>-/-</sup> macrophages.



**Fig. 3.** Levels of free cholesterol and cholesteryl ester in the wild-type and *npc1*<sup>-/-</sup> murine macrophages following treatment with mβCD-cho. Total lipids were extracted from wild-type and *npc1*<sup>-/-</sup> murine macrophages incubated with mβCD-cho for 48 hr, after which the cholesterol content was determined using the cholesterol/cholesteryl ester assay (**A**, **B**), as described in the Materials and Methods section. Phase-contrast microscopic images (**C**, upper panels) and Oil Red O-stained images (**C**, lower panels). Original magnification: ×40. The data represent the findings of two independent experiments.

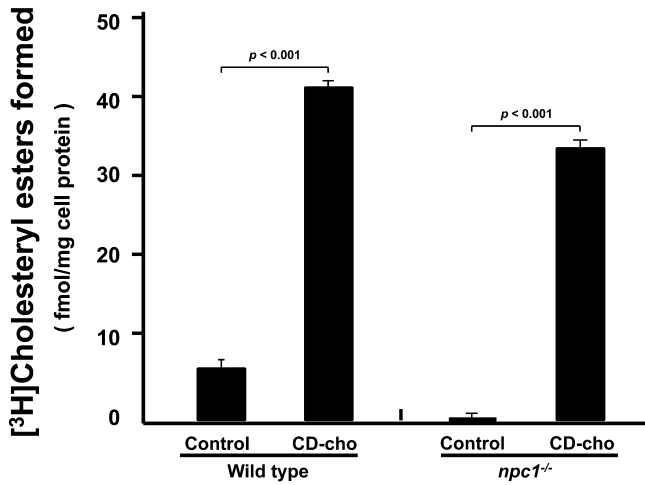
#### **Effects of mβCD-cho administration on the survival of *npc1*<sup>-/-</sup> mice**

Instead of using the conventional therapeutic approach to remove excess cholesterol using mβCD, we examined the effects of ACAT1-LE-induced cholesterol esterification on the lifespan of *npc1*<sup>-/-</sup> mice. In order to compare the effects of cholesterol removal and the induction of ACAT1-LE *in vivo*, we administered mβCD-cho or mβCD in *npc1*<sup>-/-</sup> neonate mice and evaluated their survival. As shown in Figure 5, the administration of mβCD, a simple cholesterol remover, improved the survival of these mice, as previously reported [18]. Importantly, this effect of lifespan extension obtained with mβCD is similar to the findings of a previous

report, suggesting that our experimental design is appropriate [18]. That mβCD-cho, both an ACAT1-LE inducer and cholesterol donor, also prolonged survival to that equal to that observed in the mβCD treatment group was a surprising finding. These results thus indicate that both cholesterol donor treatment and cholesterol removal treatment have similar effects on the survival of *npc1*<sup>-/-</sup> mice. The key to these results is the induction of ACAT1-LE, as shown in Figures 1–4.

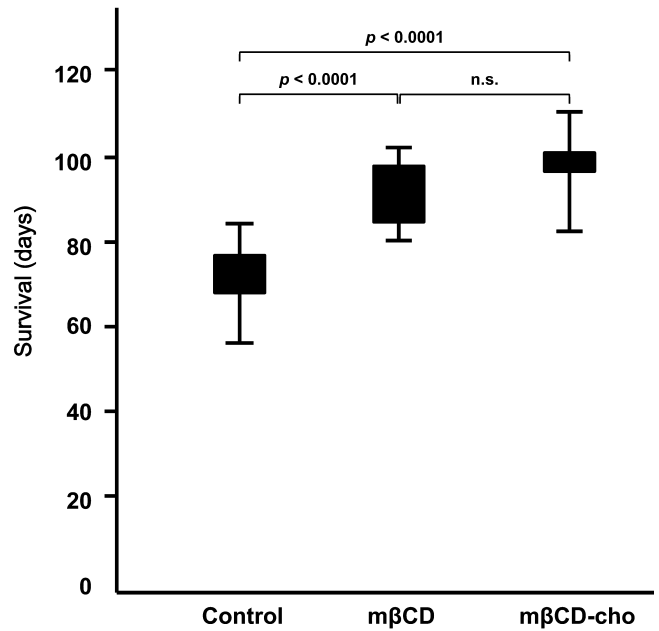
#### **IV. Discussion**

We demonstrated in this report that the induction of



**Fig. 4.** Effects of mβCD-cho treatment on cholesterol re-esterification in the wild-type and *npc1*<sup>-/-</sup> murine macrophages. Wild-type and *npc1*<sup>-/-</sup> murine macrophages treated with mβCD-cho for 48 hr were incubated with [<sup>3</sup>H]cholesteryl linoleate-labeled acetylated LDL for 24 hr, after which the amount of [<sup>3</sup>H]cholesteryl oleate was determined, as described in the Materials and Methods section. The results represent the findings of two separate experiments.

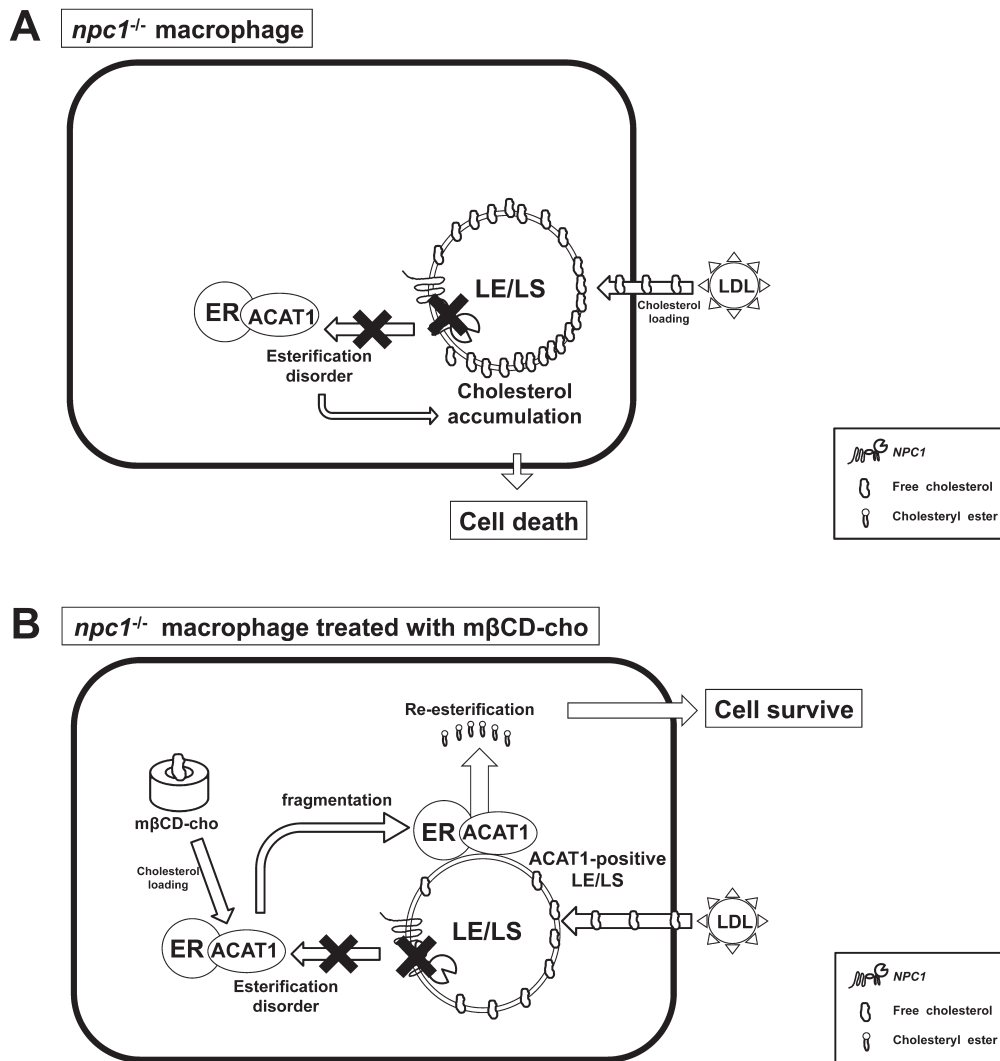
ACAT1-LE in *npc1*<sup>-/-</sup> macrophages results in the repair of intracellular cholesterol metabolism, as well as the restoration of cholesterol esterification and normalization of the intracellular free cholesterol levels. This correction also significantly improved the survival of the *npc1*<sup>-/-</sup> mice. The key issue underlying this beneficial result is the formation of ACAT1-LE, which was induced by treatment with mβCD-cho, but not acetylated LDL (Fig. 2A, B). The failure to induce acetylated LDL-mediated ACAT1-LE formation is logical because acetylated LDL is internalized via receptor-mediated endocytosis, hydrolyzed at LE/LS and transferred to the ER as a function of NPC1, where it activates ACAT1, thereby stimulating acetylated LDL-induced cholesterol esterification and ACAT1-LE formation in wild-type macrophages. In contrast, this treatment failed to produce foamy transformation or ACAT1-LE production in *npc1*<sup>-/-</sup> macrophages because it caused LE/LS to accumulate hydrolyzed free cholesterol due to the lack of NPC1 [15, 27]. Importantly, mβCD, which differs from acetylated LDL, is internalized independent of receptor-mediated endocytosis. Instead, it is internalized via pinocytosis and remains at the LE/LS, which results in ACAT1 activation and cholesterol esterification [24]. The current treatment strategy for NPC is mβCD administration in order to remove cholesterol from various cellular organelles, including the plasma membrane and LE/LS [6, 18, 29]. This strategy clearly prolongs survival in animal models via the suppression of inflammatory responses in target organs, *i.e.*, the liver and central nervous system [8, 18], and improves the clinical manifestations of NPC, even in humans [19]. Ramirez *et al.* reported that the weekly administration of mβCD prolongs the survival of diseased



**Fig. 5.** Effects of mβCD-cho administration on the survival of the *npc1*<sup>-/-</sup> mice. Saline containing mβCD-cho (250 μM of mβCD containing 16 μM of cholesterol; total volume: 100 μl) or 250 μM of mβCD; total volume 100 μl was administered to day 7 neonate *npc1*<sup>-/-</sup> mice in a single subcutaneous injection, after which the survival of the animals was determined, as described in the Materials and Methods section.

mice and normalizes the cholesterol pool in most organs, particularly the liver, although the protective effects against neurodegeneration are limited, even with this aggressive protocol [23]. The unsatisfactory neuroprotective effects achieved with multiple mβCD administration suggest that removing free cholesterol is insufficient to treat the disease.

We used a treatment strategy that differs from the conventional method for NPC. The key treatment in our experiments, rather than the usual removal of unesterified cholesterol from LE/LS, was the attainment of effective cholesterol esterification via the formation of ACAT1-LE. As demonstrated in Figure 6, mβCD-cho treatment effectively esterified free cholesterol via ACAT1-LE formation, resulting in an approximately 1.4-fold increase in survival among the *npc1*<sup>-/-</sup> mice. Our strategy was as effective as conventional mβCD treatment, thus suggesting that this alternative treatment sufficiently decreases the intracellular cholesterol level to rescue cholesterol-induced cell injury *in vivo*. However, as shown in Figure 3, mβCD-cho treatment induced significant cholesteryl ester accumulation. The intracellular cholesterol level is strictly regulated [20] (the excessive accumulation of free cholesterol harms mammalian cells, as it activates ER stress-mediated apoptosis [11]), although most mammalian cells can accumulate cholesteryl ester as lipid droplets in their cytoplasm. For this reason, mβCD-cho treatment should improve the survival of *npc1*<sup>-/-</sup> mice. Sufficient induction of ACAT1-LE may therefore be an alternative NPC treatment strategy to the



**Fig. 6.** Possible mechanism underlying ACAT1-LE-mediated cholesterol esterification in *npc1*<sup>-/-</sup> murine macrophages. (A) *npc1*<sup>-/-</sup> macrophages with impaired cholesterol esterification. (B) *npc1*<sup>-/-</sup> macrophages with ACAT1-LE and related cholesterol esterification.

conventional cholesterol removal approach. In order to confirm the validity of ACAT1-LE induction in *npc1*<sup>-/-</sup> mice *in vivo*, we are planning to assess histological improvements in neurological injury as well as hepatic damage in *npc1*<sup>-/-</sup> mice after mβCD-cho treatment in our next report.

Theoretically, conventional mβCD treatment releases unesterified cholesterol as mβCD-cho into the cytoplasm due to the high affinity of mβCD-cho for the cholesterol molecule. A previous report indicated that mβCD administration in *npc1*<sup>-/-</sup> mice activates ACAT1, resulting in significantly increased cholesteryl ester accumulation [18]. Furthermore, our preliminary experiment showed that mβCD treatment, but not mβCD-cho treatment, induces ACAT1-LE in THP-1 human macrophages following U18666A and acetylated LDL treatment, in an *in vivo* model of *npc1*<sup>-/-</sup> macrophages (data not shown). Taken together, these data suggest that conventional mβCD treat-

ment of *npc1*<sup>-/-</sup> cells also causes partial or incomplete ACAT1-LE formation, which may result in a favorable outcome of *npc1*<sup>-/-</sup> treatment. In order to clarify the details of the mechanisms underlying cholesterol esterification and ACAT1-LE formation in *npc1*<sup>-/-</sup> cells after treatment with mβCD or mβCD-cho, future experiments need to be done which should determine the optimal preparation of mβCD and mβCD-cho.

## V. Acknowledgments

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