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Cathepsin B Is Involved in the Trafficking of TNF-α-Containing Vesicles to the Plasma Membrane in Macrophages[1]

Soon-Duck Ha,*† Andrew Martins,*† Khashayarsha Khazaie,‡ Jiahuai Han,§ Bosco M. C. Chan,* and Sung Ouk Kim[2]*†

TNF-α is a potent proinflammatory cytokine, essential for initiating innate immune responses against invading microbes and a key mediator involved in the pathogenesis of acute and chronic inflammatory diseases. To identify molecules involved in the production of TNF-α, we used a functional gene identification method using retroviral integration-mediated mutagenesis, followed by LPS-stimulated TNF-α production analysis in macrophages. We found that cathepsin B, a lysosomal cysteine proteinase, was required for optimal posttranslational processing of TNF-α in response to the bacterial cell wall component LPS. Mouse bone marrow-derived macrophages from cathepsin B-deficient mice and macrophages treated with the cathepsin B-specific chemical inhibitor CA074 methyl ester or small interfering RNA against cathepsin B secreted significantly less TNF-α than wild-type or nontreated macrophages. We further showed that the inhibition of cathepsin B caused accumulation of 26-kDa pro-TNF-containing vesicles. Ectopic expression of GFP-conjugated pro-TNF further suggests that pro-TNF failed to reach the plasma membrane without intracellular cathepsin B activity. Altogether, these data suggest that intracellular cathepsin B activity is involved in the TNF-α-containing vesicle trafficking to the plasma membrane. ***The Journal of Immunology,*** **2008, 181: 690–697.**

Production of the potent inflammatory cytokine TNF-α in response to invading microbes is a crucial step for mounting initial innate immune responses. However, uncontrolled production of TNF-α is linked to the pathogenesis of severe acute and chronic inflammatory diseases (1). Thus, the biosynthesis and release of TNF-α should be tightly regulated at different levels to prevent inadvertent production under normal condition (1–4). Activation of macrophages by the bacterial cell wall component LPS is mediated through TLR4, resulting in the recruitment of signaling adaptors such as MyD88 and TRIF (5). This recruitment of signaling adaptors to TLR4 induces activation of signaling cascades involving multiple signaling molecules, including the family of protein serine kinases IL-1R-associated kinase 1 and kinase 4 and the adaptor molecule TNFR-associated factor 6. Subsequent activation of MAPKs and transcription factor NF-κB occurs, which controls transcription of cytokines including TNF-α (5). Additional layers of TNF-α production control occur at the level of translation. TNF-α mRNA contains an AU-rich element in its 3′ untranslated region, which determines its half-life and translational efficiency through binding tristetraprolin or T cell intracellular Ag-1 that promotes mRNA degradation or inhibits protein translation, respectively (6).

TNF-α is encoded as a 26-kDa type II transmembrane precursor (pro-TNF), which is transported from the *trans*-Golgi network to the recycling endosome (7), then delivered to the cell surface where pro-TNF undergoes proteolytic cleavage by the TNF-α-converting enzyme (8–11). Membrane fusion between the TNF-α-containing vesicles from *trans*-Golgi network and the recycling endosomes, and between the recycling endosomes and the cell surface membrane was shown to be mediated through interactions between various *trans*-SNARE (soluble-*N*-ethylmaleimide-sensitive factor-attachment protein (SNAP)[3] receptor) family members (4). Recent studies have shown that TNF-α surface delivery and secretion is regulated through a process involving cholesterol-dependent lipid raft formation at the phagocytic cup of activated macrophages (7, 12); however, it is unknown how TNF-α surface delivery is regulated and whether it can be facilitated upon TLR activation. We used a functional gene identification procedure using retrovirus integration-generated mutagenesis (13–15) and identified that cathepsin B activity was required for optimal TNF-α transportation to the plasma membrane. Cathepsin B is a lysosomal cysteine protease involved in the degradation of cellular proteins in lysosomes. Unlike other cathepsins, cathepsin B functions as an endopeptidase at neutral pH and is also found in extralysosomal sites, including the cytosol, the plasma membrane, and pericellular spaces, where it participates in various cellular processes including cancer metastasis, inflammation, myoblast differentiation, IL-1β production, and cell death (16–21). In this study, we demonstrate that cathepsin B is involved in the posttranslational process of TNF-α, likely in the trafficking of TNF-α-containing vesicles to the plasma membrane.

*Department of Microbiology and Immunology, and †Infectious Diseases Research Group, Siebens-Drake Research Institute, University of Western Ontario, London, Ontario, Canada; ‡Department of Microbiology-Immunology, Northwestern University, Chicago, IL 60611-3015; and §The Scripps Research Institute, La Jolla, CA 92037

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[2] Address correspondence and reprint requests to Dr. Sung Ouk Kim, Infectious Diseases Research Group, Siebens-Drake Research Institute, Room 119, University of Western Ontario, 1400 Western Road, London, Ontario N6G 2V4, Canada. E-mail address: sung.kim@schulich.uwo.ca

[3] Abbreviations used in this paper: SNAP, soluble-*N*-ethylmaleimide-sensitive factor-attachment protein; SNARE, SNAP receptor; siRNA, small interfering RNA; BMDIM, bone marrow-derived immortalized macrophage.

Materials and Methods

Materials and reagents

Cathepsin B inhibitor III, cathepsin B inhibitor IV, cathepsin K inhibitor III, cathepsin G inhibitor I, and calpain inhibitor III (zVF-Cho) were purchased from Calbiochem (EMD Bioscience). Cathepsin B and cathepsin L inhibitor (zFF-fmk) was purchased from Sigma-Aldrich. Abs for p38 MAPK and cathepsin B were obtained from Cell Signaling Technology and Calbiochem, respectively. Mouse and human TNF-α Abs were purchased from eBioscience.

Cell culture

Mouse bone marrow-derived immortalized macrophages (BMDIM) were prepared as previously described (22–24) from C57BL/6 mice. Bone marrow-derived macrophages from cathepsin B gene (*Ctsb*)-deficient ($Ctsb^{-/-}$) or control strain C57BL/6 ($Ctsb^{+/+}$) mice or from the human monocytic cell line THP-1 were cultured in RPMI 1640 medium containing 10% heat-inactivated FBS (Sigma-Aldrich), 10 mM MEM nonessential amino acid solution, 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 1 mM sodium pyruvate. Cells were grown at 37°C in the humidified atmosphere of 5% CO_2. Human PBMC were isolated from whole blood as previously described (25).

Mutagenesis with retrovirus, screening for defects in TNF-α production, and identification of retroviral targeting gene

BMDIM were transfected with the pDisrup8-loxP retroviral vector containing a blasticidin resistance gene as previously described (15), except a loxP sequence was incorporated into the vector. Each blasticidin resistance clone was cultured for 2 wk and tested for TNF-α production in response to LPS (1 μg/ml). TNF-α bioassay was performed as previously described (26). To rescue retroviral insertion-induced mutations, $CTSB^{mut}$, a partial mRNA sequence of the fused gene product, was transfected with a retroviral vector expressing Cre (pBaBe-Cre) and puromycin resistance gene. Cre-expressing cells were then selected by culturing cells in the presence of puromycin (10 μg/ml). All puromycin resistance cells became sensitive to blasticidin. Identification of the gene targeted by pDisrup8pLox was performed using 3′-RACE as previously described (15). Primers used for semiquantitative analysis for pro-retrovirus, cathepsin B, and GAPDH are as follow: pro-retrovirus (BSR4) 5′-GTGAAGGACAGTGATGGAC AG-3′ and cathepsin B (antisense) 5′-GCCCTAAGGACTGGACAATGA-3′; cathepsin B (sense) 5′-TGCAGGCCCAGGCTGTCG-3′ and (antisense) 5′-GCCCTAAGGACTGGACAATGA-3′; and GAPDH (sense) 5′-GCATTGTGGAAGGGCTCATG-3′ and (antisense) 5′-TTGCTGTTG AAGTCGCAGGAG-3′.

Measurement of cathepsin B activity

Cathepsin B activity was measured by cathepsin B activity assay kit (BioVision). Briefly, cells were washed with the PBS, and lysed in chilled cathepsin B cell lysis buffer. After incubation cell on ice for 10 min, 10 mM cathepsin B substrate (Ac-RR-AFC, 200 μM final concentration) was added and incubated at 37°C for indicated time. The release of free amino-4-trifluoromethyl coumarin was monitored in a fluorometer (Fluoroskan ascent FL; Thermo LabSystems) with a 409 nm excitation filter and 515 nm emission filter.

Quantitative real-time PCR

mRNA expression of mouse TNF-α in macrophages was quantified on the Rotor-Gene RG3000 quantitative multiplex PCR instrument using the Brilliant SYBR Green PCR Master mix (Applied Biosystems). Total Cellular RNA was isolated using TRIzol (Life Technologies) according to the manufacturer's instructions. Briefly, 4 μg of total RNA was reverse transcribed by using oligo(dT) primers and the Superscript II reverse transcriptase system (Invitrogen) according to the manufacturer's recommendations. Oligonucleotide primers used were the following: for mouse TNF-α, 5′-CTGGAAATAGCTCCCAGAA-3′ (5′ primer) and 5′-CATTTGGGAACT TCTCATCC-3′ (3′ primer); and for GAPDH, 5′-GCATTGTGGAAGGGC TCATG-3′ (5′ primer) and 5′-TTGCTGTTGAAGTCGCAGGAG-3′ (3′ primer).

Small interfering RNA (siRNA)

siRNA oligonucleotides directed against human cathepsin B were purchased from Dharmacon. Transfection of THP-1 cell with siRNAs was performed using the Nucleofector II kit (Amaxa Biosystems), according to the manufacturer's instructions. Briefly, cells were subcultured 1 day before nucleofection. The next day, cells were harvested, resuspended in room temperature Nucleofector solution V to a final concentration of 2.0×10^6 cells/100 μl, and mixed with siRNA (Dharmacon), for human cathepsin B (NM_001908, ON-TARGET Plus Duplex J-004266-13-0005). Transfection was performed using the Nucleofector I device (Amaxa Biosystems). At 48 h after nucleofection, cells were plated and stimulated with LPS for TNF-α assay. Other cells were harvested for cathepsin B protein knockdown analysis. The verification of cathepsin B knockdown was performed by Western blot analysis using anti-human cathepsin B Ab (eBioscience).

Total cell lysate preparation and immunoblotting

Total cell lysate preparation and immunoblotting procedures were performed as previously described (25). Briefly, cells were lysed in ice-cold lysis buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1 mM Na_3VO_4, 40 mM β-glycerophosphate, 30 mM NaF, and 20 mM sodium pyrophosphate (pH 7.2)) containing a protease inhibitor cocktail (Roche). The cell lysates were incubated on ice for 10 min and centrifuged at 12,500 rpm for 15 min at 4°C. The supernatants were electrophoretically resolved in SDS-polyacrylamide gels, followed by transfer onto nitrocellulose membranes (Bio-Rad). The membranes were blocked at room temperature for 1 h with 5% (w/v) skim milk, and then incubated overnight at room temperature with the primary Ab. The membranes were washed and developed using an ECL detection system (Pierce).

Metabolic radiolabeling and immunoprecipitation

BMDIM were plated 2.5×10^6 cell/well. Cells pretreated with or without the cathepsin B inhibitor CA074 methyl ester (CA-Me) for 45 min were stimulated with LPS 1 μg/ml for 90 min. LPS-stimulated cells, or untreated cells as the control, were replaced with methionine-free medium for 60 min under the LPS (1 μg/ml). They were then labeled for 1 h with 200 μCi/ml of [^{35}S]methionine (PE Life and Analytical Science). The labeled cells were washed and replaced with fresh regular growth medium. At the time interval indicated, medium and cells were collected. Cells were lysed using ice-cold lysis buffer (20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 1 mM Na_3VO_4, 40 mM β-glycerophosphate, 30 mM NaF, and 20 mM sodium pyrophosphate (pH 7.2)) containing protease inhibitor cocktails (Roche). TNF-α was immunoprecipitated from the samples, using anti-mouse TNF-α Ab (eBioscience), separated on SDS-polyacrylamide gels and visualized by autoradiography.

Flow cytometry analysis

BMDIM were treated with or without cathepsin B inhibitors for 30 min before stimulation with LPS (100 ng/ml) for 18 h. Cells were then fixed in 1% formalin for 10 min with periodic agitation. After two washes with PBS, cells were then resuspended and incubated for 30 min on ice in PBS containing 0.1% saponin and 10% FBS. TNF-α was then stained using a PE-labeled anti-mouse TNF-α Ab (eBioscience) for 30 min. After two final washes, cells were resuspended to 1 million cells/ml in PBS and analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Data analysis was performed using CellQuest software (BD Biosciences).

Immunofluorescence staining and TNF-α trafficking analysis

BMDIM were electroporated with the plasmid endcoding EGFP-TNF (obtained from Dr. J. Stow (University of Queensland, Brisbane, Australia). Transfected cells were fixed with 4% paraformaldehyde and observed through Bio-Rad Radiance 2000 Two-Photon fluorescence confocal microscopy. Images were obtained using LaserSharp 2000 software. The fixed macrophages were also stained with 10 μg/ml Hoechst 33258 (Sigma-Aldrich) in PBS for 2 min to visualize nuclear staining and followed by image analysis under confocal microscopy. For immunofluorescence staining, macrophages were permeabilized with 0.1% Triton X-100 and immunostained for TNF-α. Endogenous TNF-α was detected by immunofluorescence using biotinylated anti-rabbit IgG and fluorescein avidin D (Vector Laboratories), using a Qimaging (Burnaby) cooled charged-coupled device camera on an Axioscope 2 (Carl Zeiss) microscope.

Results

Disruption of Ctsb *in macrophages causes a defect in TNF-α production*

To identify genes involved in the production of TNF-α in response to LPS, we used retrovirus-mediated mutagenesis in BMDIM. The retroviral vector was constructed as previously described (15), except that the loxP site was incorporated at the 3′ long terminal repeat, which is duplicated during chromosomal insertion (Fig. 1*A*).

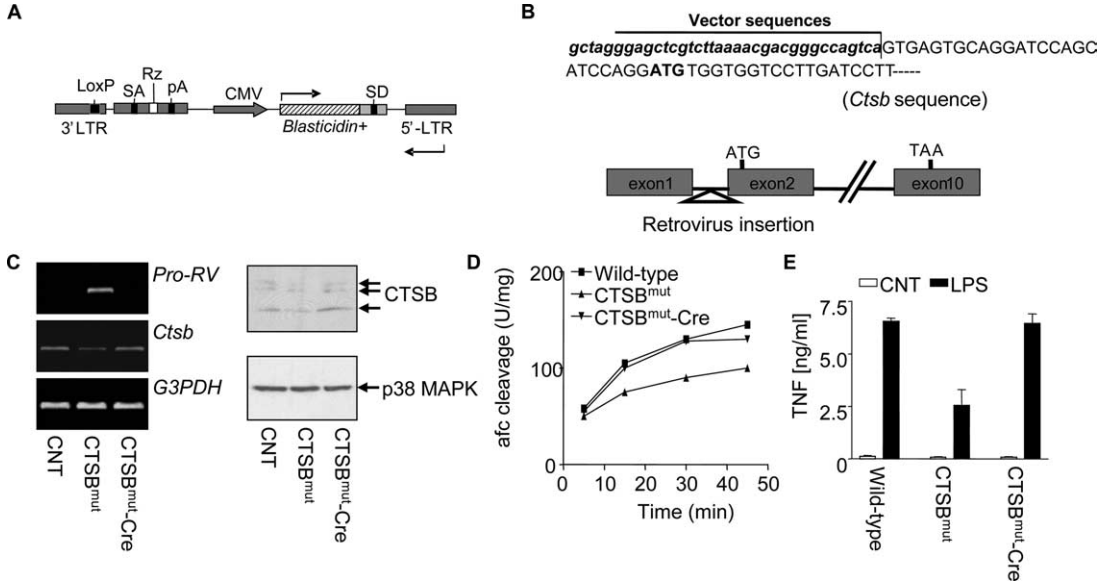

FIGURE 1. Disruption of *Ctsb* in macrophages confers defect in TNF-α production. *A*, Diagram of the retroviral vector pDisrup8-loxP used for *Ctsb* disruption. Rz, Ribozyme sequence; SD, splicing donor; SA, splicing acceptor; pA, poly(A) signal sequence; CMV, CMV promoter; *Blasticidin+*, blasticidin-resistant gene; LTR, long terminal repeat. *B*, Identification of retroviral vector targeting gene. Retroviral vector was inserted in between exon 1 and exon 2 of *Ctsb* by 3′-RACE and DNA sequencing as described in *Materials and Methods*. *C*, Generation of null allele of cathepsin B by retroviral vector and its rescue by pCre. Semiquantitative RT-PCR was performed (*left*) using a primer targeting the retroviral vector and a cathepsin B-specific primer (*upper panel*) or both primers targeting the *Ctsb* (*middle panel*). GAPDH was amplified as a control using mouse GAPDH-specific primers (*lower panel*). CTSB^{mut}, a clone containing cathepsin B-null allele generated by retroviral vector, and CTSB^{mut}-Cre, a clone cured cathepsin B-null allele by Cre macrophages were treated. Western blot analysis (*right*) was performed in total cell lysates from wild-type, CTSB^{mut}, or CTSB^{mut}-Cre macrophages for cathepsin B and p38 MAPK (for loading control). *D*, Cathepsin B activity in wild-type, CTSB^{mut}, and CTSB^{mut}-Cre macrophages was measured in total cell lysates as described in *Materials and Methods*. *E*, Similarly, these cells were treated with LPS (100 ng/ml) for 6 h and TNF-α in the cell culture medium was measured by TNF-α bioassay. Data are expressed as mean ± SD (n = 3 experiments).

The proviral sequences can readily be excised out from chromosome by ectopically expressing Cre recombinase. This feature greatly enhances the convenience in validating the effects of viral insertion because excision of provirus should cure the defect incurred by a viral integration. As described in *Materials and Methods*, we screened ~1000 blasticidin-resistant retrovirus-mutated clones and identified a clone defective in producing TNF-α in response to LPS. By 3′-RACE of the mRNA fused with blasticidin, the disrupted gene was identified as *Ctsb*. A partial mRNA sequence of the fused gene product generated by retroviral insertion in the clone (termed CTSB^{mut}) is shown in Fig. 1*B*. The blasticidin gene encoded by the viral vector appeared to be inserted in the intron between exon 1 and exon 2 and, therefore, disrupted *Ctsb* at one allele. Expression of Cre using a retroviral vector containing the puromycin resistance gene in CTSB^{mut} rendered the cells puromycin-resistant but sensitive to blasticidin (data not shown), and the *Ctsb*-fused blasticidin gene mRNA was no longer expressed in the Cre-expressing CTSB^{mut} clone (CTSB^{mut}-Cre) (Fig. 1*C*, *left top panel*). Based on semiquantitive RT-PCR analysis, the level of cathepsin B mRNA was diminished in CTSB^{mut}, and wild-type mRNA levels were restored by expressing Cre (CTSB^{mut}-Cre) Fig. 1*C*, *left middle panel*). Similarly, the level of cathepsin B protein in CTSB^{mut} cells decreased to ~50% of the level found in wild-type or CTSB^{mut}-Cre cells (Fig. 1*C*, *right*). We further confirmed that total cathepsin B activity and TNF-α production induced by LPS in CTSB^{mut} were also reduced to ~50% of wild-type cells, and returned to normal levels after excising viral insertion by CRE (CTSB^{mut}-Cre) (Fig. 1*D*). These results indicate that the decrease in TNF-α production and cathepsin B activity in CTSB^{mut} were due to a retrovirus-mediated gene disruption.

Cathepsin B activity is involved in the posttranslational processing of TNF-α in macrophages

To further verify the involvement of cathepsin B in TNF-α production, bone marrow-derived macrophages from wild-type (C57BL/6) and cathepsin B deficient (C57BL/6$^{ctsb-/-}$) mice were treated with various times and doses of LPS, and the levels of TNF-α production were analyzed. *Ctsb*$^{-/-}$ macrophages produced significantly lower levels of TNF-α in response to LPS than similarly treated wild-type macrophages (*Ctsb*$^{+/+}$) (Fig. 2*A*), even though the level of TNF-α mRNA was increased in the same extent by LPS (Fig. 2*B*, *bottom*). Consistent with these results, there was no difference in the induction of mRNA levels in wild-type, CTSB^{mut}, and CTSB^{mut}-Cre cells (Fig. 2*B*, *top*). To further examine whether the defect in TNF-α production in cathepsin B mutant macrophages is TLR4-specific or if it applies to stimulation of other TLRs, we treated wild-type, CTSB^{mut}, and CTSB^{mut}-Cre cells with TLR agonists lipoteichoic acid for TLR2 and CpG for TLR9. In all treatments, the levels of TNF-α production in CTSB^{mut} were significantly lower than production levels found in either wild-type or CTSB^{mut}-Cre cells (Fig. 2*C*).

TNF-α production in human monocytes is more sensitive to CA-Me than are murine macrophages

We examined the involvement of cathepsin B in human monocytic cell line THP-1 using siRNAs against cathepsin B. THP-1 cells treated with the siRNA against cathepsin B down-regulated the expression of cathepsin B ~50% of scrambled siRNA-treated (control) cells. The production of TNF-α in response to LPS (100 ng/ml) was diminished to a similar extent (~50%) by treatments using siRNAs against cathepsin B (Fig. 3*A*). Because *Ctsb*$^{-/-}$

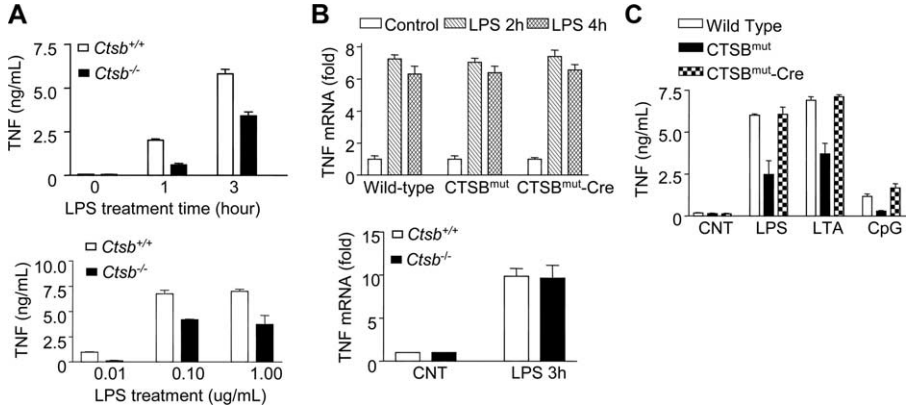

FIGURE 2. Macrophages deficient in cathepsin B show defects in TNF-α production in response to inflammatory stimuli. *A*, Bone marrow-derived macrophages from wild-type C57BL/6 ($Ctsb^{+/+}$) or $Ctsb^{-/-}$ mice were stimulated with LPS (1 μg/ml) and production of TNF-α was measured in culture medium after the time indicated in the experiment (*top*). Similarly, production of TNF-α was measured after treating cells with various doses of LPS as indicated in the experiment for 3 h (*bottom*). *B*, Macrophages wild-type or deficient in cathepsin B were treated with LPS (1 μg/ml) for 3 h and TNF-α mRNA levels were analyzed by real-time RT-PCR. *C*, Wild-type, $CTSB^{mut}$, or $CTSB^{mut}$-Cre cells were treated with LPS (100 ng/ml), lipoteichoic acid (LTA; 10 μg/ml) or CpG (20 μg/ml) for 5 h and TNF-α production in culture medium was measured by TNF-α bioassay. Data are expressed as mean $\pm$ SD ($n = 3$ experiments).

macrophages showed significant but partial defects in TNF-α production, we used the membrane permeable cathepsin B-specific inhibitor CA-Me to compare the extent of cathepsin B dependence in TNF-α production between mouse bone marrow-derived and human THP-1 macrophages. CA-Me inhibited TNF-α production in a dose-dependent manner, resulting in ~50% inhibition of TNF-α secretion at 5 μM, ~80% at 50 μM, and >95% at 100 μM in THP-1 cells (Fig. 3*B*, *left*). In comparison, mouse bone marrow-derived macrophages were less sensitive to the inhibitors, showing ~50% reduction even at a maximum concentration of 100 μM CA-Me (Fig. 3*B*, *right*). CA-Me at the concentration of 100 μM had no further suppressive effects on TNF-α production in $Ctsb^{-/-}$ murine macrophages (Fig. 3*B*, *right*), confirming that the TNF-α suppressive effect of CA-Me was specifically due to inhibition of cathepsin B activity at this concentration. Cathepsin B and cathepsin L inhibitor, zFF-fmk had similar inhibitory effects on TNF-α production as CA-Me, but other cathepsin or calpain inhibitors failed to inhibit TNF-α production in either wild-type or $Ctsb^{-/-}$ macrophages (Fig. 3*B*, *right*). In human PBMC, CA-Me at 50 μM inhibited TNF-α secretion to ~80% similar to the level of inhibition elicited by a protein transport inhibitor brefeldin A (Fig. 3*C*).

Cathepsin B activity is required for the posttranslational processing of TNF-α

Because inhibition or knockdown of cathepsin B had no effects on TNF-α mRNA levels (Fig. 2*B*), we examined whether cathepsin B was involved in translation or secretion of TNF-α. To examine whether cathepsin B is involved in the translation of TNF-α in BMDIM, pro-TNF (26-kDa) protein was immunoprecipitated after radiolabeling cells were pulsed [^{35}S]methionine for 1 h and chased for the next 90 min in the presence or absence of CA-Me. No significant difference in the levels of newly synthesized pro-TNF

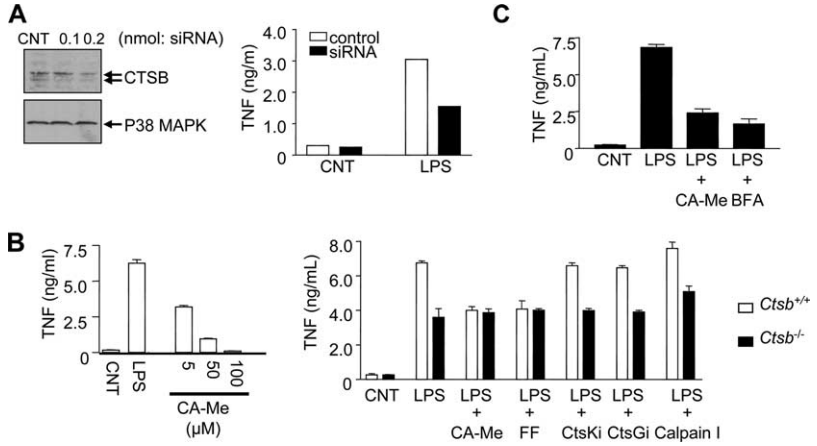

FIGURE 3. TNF-α production in human monocytes is more sensitive to the CA-Me than murine macrophages. *A*, Human monocytic cell line THP-1 cells were transfected with siRNA against cathepsin B (siCTSB) or scrambled siRNAs (CNT) for 48 h. Cells were then plated on a new 24-well plate and stimulated with LPS (1 μg/ml) for 5 h. Cell culture medium was collected for TNF-α assay and cells were used for Western blot analysis for cathepsin B and p38 MAPK for loading control. *B*, THP-1 cells pretreated (*left*) with various concentrations of CA-Me for 30 min were stimulated with LPS (1 μg/ml) for 5 h. Similarly, bone marrow-derived macrophages from wild-type C57BL/6 ($Ctsb^{+/+}$) and $Ctsb^{-/-}$ mice were pretreated (*right*) with CA-Me (100 μM), cathepsin B and cathepsin L inhibitor zFF-fmk (FF; 50 μM), cathepsin K inhibitor (CtsKi; 50 μM), cathepsin G inhibitor (CtsGi; 50 μM), and calpain inhibitor I (50 μM) for 30 min and then stimulated with LPS (1 μg/ml) for 3 h. *C*, Freshly isolated human PBMC were treated with CA-Me (50 μM) or brefeldin A (BFA) for 30 min and then stimulated with LPS (1 μg/ml) for 5 h. *B* and *C*, TNF-α in culture medium was measured by bioassay. Data are expressed as mean $\pm$ SD of over three experiments.

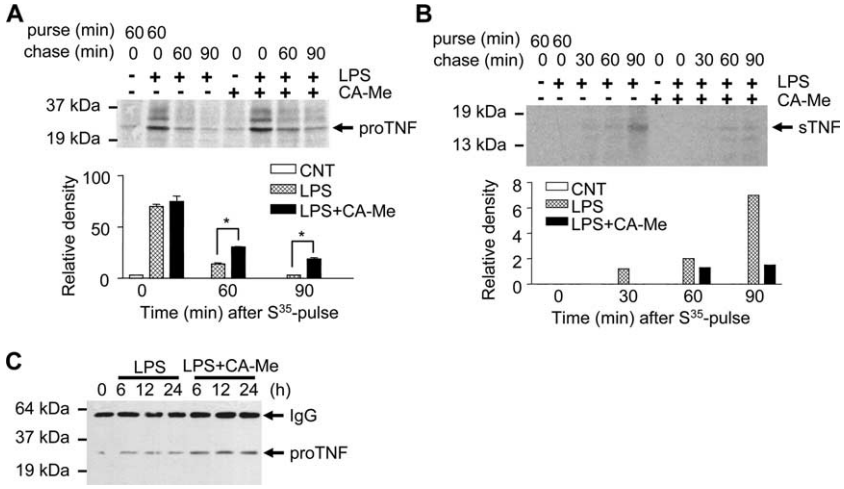

FIGURE 4. CTSB inhibition induced intracellular accumulation of pro-TNF. *A* and *B*, BMDIM pretreated with or without CA-Me (50 μM) for 45 min were treated with LPS (1 μg/ml) for 90 min and then metabolically labeled with [^{35}S]methionine for 60 min. Cell lysates and culture medium were collected at times 0, 60, and 90 min after [^{35}S]methionine labeling. Labeled TNF-α in cell lysates (*A*) and culture medium (*B*) were immunoprecipitated and visualized by autoradiography. The intensity of the bands was analyzed using the NIH Image program. Data are expressed as mean ± SD (n = 2 experiments). *C*, BMDIM pretreated with or without CA-Me (50 μM) were treated with LPS (1 μg/ml) for the times indicated in the experiment, and TNF-α was immunoprecipitated and blotted with TNF-α Ab.

was detected between CA-Me-treated and nontreated cells (Fig. 4*A*, *second* and *sixth lanes*). However, labeled pro-TNF was detected for significantly longer time periods in CA-Me-treated than in nontreated cells (Fig. 4*A*, *bottom*). At the same time, the cleaved soluble form of radiolabeled 17-kDa TNF-α was detected at significantly higher levels in nontreated than in CA-Me-treated cell culture medium (Fig. 4*B*). Consistently, Western blot analysis for the overall amounts of intracellular pro-TNF indicated that CA-Me-treated cells contained higher levels after 6 h (Fig. 4*C*). A higher level of intracellular TNF-α in CA-Me-treated cells was confirmed by ELISA of cell lysates (data not shown). These results suggest that cathepsin B is involved in posttranslational TNF-α production steps, either in TNF-α-containing vesicle trafficking to the plasma membrane or in the release of TNF-α from the plasma membrane into culture medium.

TNF-α-containing cargo vesicles fail to reach the plasma membrane in the absence of cathepsin B

To visually examine where the defect in TNF-α production originates in the absence of cathepsin B, TNF-α was immunostained in cells treated with LPS with or without CA-Me. LPS alone slightly increased TNF-α staining inside cells in a distinct puncta (Fig. 5*A*). However, TNF-α staining was further enhanced inside cells treated with LPS together with CA-Me. The TNF-α-specific staining was highly localized at a focal point close to the plasma membrane but was absent from the plasma membrane. These data suggest that cathepsin B is involved in trafficking TNF-α-containing vesicle, rather than cleavage of pro-TNF on the surface of cell membrane. However, it is still possible that the high intensity of TNF-α immunofluorescence was due to an accumulation of uncleaved pro-TNF recycled into cytosol because uncleaved pro-TNF is endocytosed and appears in intracellular vesicles (27). To address this question, we ectopically expressed N-terminal GFP-conjugated pro-TNF in BMDIM in the presence or absence of CA-Me. GFP conjugation to the N terminus of pro-TNF allows GFP to remain within the plasma membrane after cleavage of the C-terminal portion of pro-TNF by TNF-α-converting enzyme. Therefore, the presence of GFP in the plasma membrane indicates that pro-TNF has reached the plasma membrane. As expected, GFP was detected within the cytosol as distinct puncta and highly detected on the plasma membrane after 8 h of transient transfection of GFP-conjugated pro-TNF (Fig. 5*B*). However, in cells treated with CA-Me, GFP was only detected in cytosol in distinct puncta and absent in the plasma membrane even 16 h posttransfection. Most CTSB^{mut} cells also showed an accumulation of GFP in cytosol in puncta but much less GFP association with the plasma membrane, whereas Cre-expressing CTSB^{mut}-Cre clones showed GFP associated with the plasma membrane (Fig. 5*C*). Similar results were also obtained

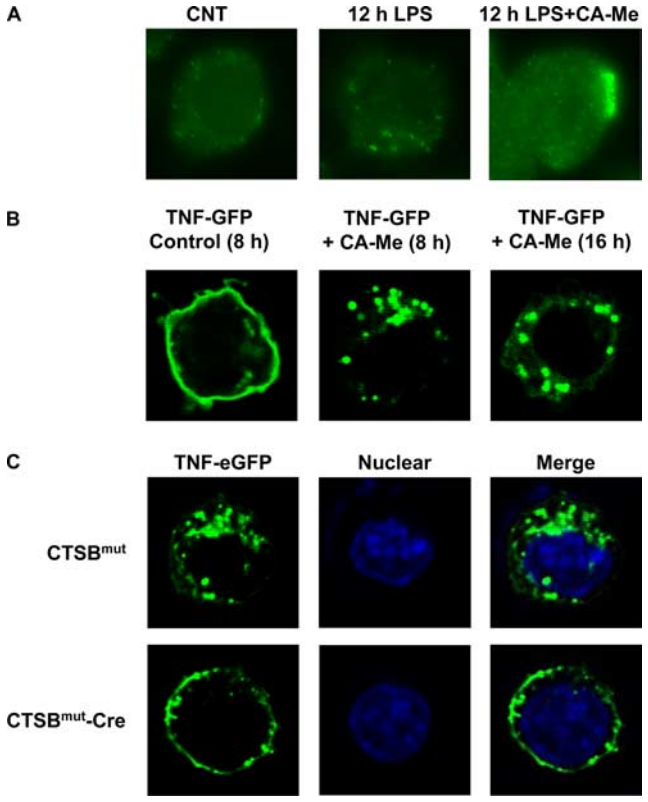

FIGURE 5. Cathepsin B is involved in trafficking of TNF-α-containing vesicles to the plasma membrane. *A*, Intracellular immunostaining of endogenous TNF-α. BMDIM grown on coverslips were treated with LPS in the absence or presence of CA-Me (50 μM) for 12 h or with scrambled siRNA (control) cells (CNT). Cells were then fixed, immunostained with TNF-α Ab, and visualized using an Axioscope 2 microscope. TNF-α-containing vesicles were strongly detected in a distinct puncta at a focal point close to plasma membrane only in cells treated with CA-Me. *B*, N-terminal GFP-conjugated pro-TNF was transiently transfected in the absence or presence of CA-Me (50 μM) for 8 or 16 h in BMDIM and visualized using a confocal microscope. *C*, Similarly, CTSB^{mut} or CTSB^{mut}-Cre were transiently transfected with N-terminal-conjugated pro-TNF. After 8 h, cells were stained with Hoechst 33258 dye for nuclear staining, and GFP-conjugated TNF-α and nucleus were visualized using a confocal microscope.

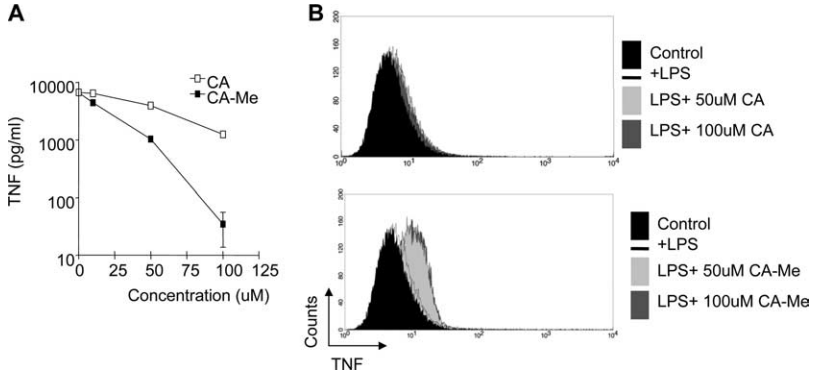

FIGURE 6. Intracellular cathepsin B activity is required for trafficking of TNF-α-containing vesicles to the plasma membrane. *A*, BMDIM pretreated with various concentrations of CA074 or CA-Me for 30 min were stimulated by LPS (100 ng/ml) for 5 h. TNF-α presented in cell culture medium were analyzed using bioassay. Data are expressed as mean $\pm$ SD ($n = 3$ experiments). *B*, BMDIM were pretreated with CA074 or CA-Me (50 or 100 μM) for 30 min and treated with LPS (100 ng/ml) for 18 h. The presence of intracellular TNF-α was measured using FACS analysis. Dat shown are representative results of similar observations in three independent experiments.

in *Ctsb*$^{+/+}$ and *Ctsb*$^{-/-}$ bone marrow-derived macrophages, respectively (data not shown). These results suggest that cells lacking cathepsin B have a defect in transporting GFP-conjugated pro-TNF to the plasma membrane.

Intracellular cathepsin B activity is required for TNF-α secretion

Cathepsin B can be secreted to pericellular compartments and affect TNF-α release from the plasma membrane. To examine whether pericellular cathepsin B activity was involved in the process of TNF-α release, we compared the effects of two cathepsin B inhibitor homologs, membrane permeable CA-Me with the less permeable form of the inhibitor CA074. CA-Me is a proinhibitor, which is converted to CA074 after internalization (28). The effectiveness of CA-Me in suppressing TNF-α production was much greater than that of CA074 (Fig. 6*A*). We further analyzed the accumulation of TNF-α in the cytosol using flow cytometry. In agreement with fluorescence microscopy and Western blot data (Figs. 4 and 5), newly synthesized TNF-α was quickly released from cells, resulting in only slight intracellular accumulation of TNF-α in either LPS alone or LPS plus CA074-treated (50 μM) cells. In contrast, CA-Me enhanced intracellular TNF-α accumulation over an 18-h period (Fig. 6*B*), suggesting that intracellular cathepsin B activity in the cytosol, rather than in pericellular space, is likely involved in TNF-α secretion.

Discussion

To identify molecules that regulate TNF-α synthesis and secretion, we used a retrovirus-mediated random mutagenesis in macrophages. Each mutant clone was analyzed for TNF-α production in response to LPS treatment. This procedure identified that cathepsin B was required for optimal production of TNF-α in response to a TLR4 ligand LPS (Fig. 1). We further confirmed that cathepsin B is also required for TNF-α production in response to a TLR2 ligand lipoteichoic acid and TLR9 ligand CpG (Fig. 2*C*) at posttranslational levels (Fig. 2*B*) and in both mouse and human macrophages or monocytes (Fig. 3).

Cathepsin B is primarily known as a lysosomal cysteine protease involved in the degradation of cellular proteins, bone resorption, trypsinogen activation, and cell death (29, 30). Recent studies further showed that cathepsin B is released to other sites of cells and involved in various cellular functions. Cathepsin B is secreted to pericellular environments where it enhances tumor cell invasiveness (16), myoblast differentiation (17), and survival of cytotoxic lymphocytes after degranulation (18). In certain conditions, cathepsin B is released to cytosol and triggers arachidonic acid release (19), caspase-1 or caspase-11 activation (20, 31), and tumor cell invasion (21). A rise in intracellular cathepsin B activity has been observed long ago in peritoneal macrophages or human monocytic cell line THP-1 cells in responses to endocytosis (32) or inflammatory stimuli such as LPS and IFN-γ (33, 34). We show in this study that cathepsin B is involved in the posttranslational regulation of TNF-α, likely in the trafficking of TNF-α containing vesicles to the plasma membrane. Based on the results showing much higher sensitivity to the membrane permeable version of CA-Me than to the less-permeable homolog CA074 in TNF-α secretion (Fig. 6), intracellular rather than pericellular cathepsin B activity is likely involved in TNF-α secretion. In *Ctsb*$^{-/-}$- or CA-Me-treated macrophages, the ectopic expression of GFP-conjugated pro-TNF showed a defect in reaching cell surface, further substantiating the notion that cathepsin B is not involved in the cleavage of pro-TNF or TNF-α-converting enzyme at the cell surface (Fig. 5). However, this study could not delineate whether the site of cathepsin B action was in the cytoplasm, lysosomes, or another intracellular compartment. Cathepsin B was shown to be released into the cytosol in certain tumor cells (21) and cells treated with different stimuli such as TNF-α or the *Streptomyces* toxin nigericin (19, 20) and in cells infected with parvovirus H-1 (35). Although total cathepsin B activity was not changed by LPS within 5 h of the treatment, we detected a small amount of cathepsin B released into the cytosol as early as 30 min of LPS treatments, based on Western blot analysis (data not shown). However, further definitive studies are required to conclude whether cytosolic cathepsin B is required for the fusion of TNF-α-containing vesicles to the plasma membrane.

Recent studies have elucidated that the newly synthesized pro-TNF is initially accumulated in the Golgi complex (36). Pro-TNF-containing vesicles are then transported from the *trans*-Golgi network to the recycling endosome and subsequently to the cell surface through two distinct membrane fusion processes (7, 37, 38). The first fusion process is mediated by Q-SNAREs, comprising syntaxin 6, syntaxin 7, and Vit1b (vesicle transport through interaction with t-SNAREs homolog 1b), of the Golgi-complex TNF-α carrier vesicle and the R-SNARE VAMP3 (vesicle-associated membrane protein 3) of the recycling endosome. The second fusion process is through interaction between the VAMP3 of the recycling endosome and the Q-SNARE complex of the plasma membrane, comprising synthaxin 4 and SNAP-23. Activation of macrophages by LPS induces expression of Q-SNARE components synthaxin 4 and SNAP-23 to accommodate the increased trafficking during TNF-α secretion (38). Cathepsins such as cathepsin L (39) or cathepsin B (40, 41) can be localized in the nucleus and regulate transcription factors. We did not detect differences in the levels of synthaxin 4 and SNAP-23 in CA-Me-treated or *Ctsb*$^{-/-}$ macrophages (data not shown), suggesting that cathepsin B is not inhibiting fusion of TNF-α-containing vesicles to the plasma membrane by down-regulating these

SNARE components. Cathepsin B has also shown to be involved in both transcription and posttranslational protein processes such as prorenin (42), thyroglobulin (43), and other proteinases (44). Therefore, it is possible that cathepsin B regulates TNF-α vesicle trafficking through regulating SNARE components either at transcriptional or posttranslational levels.

Based on results from *Ctsb*$^{-/-}$ macrophages (Fig. 2*A*), TNF-α can be secreted through a cathepsin B-independent pathway and the extent of cathepsin B involvement in TNF-α secretion appears to be varied in different macrophages. In THP-1 and primary human monocytic cells, the cathepsin B-specific inhibitor CA-Me almost completely blocked TNF-α production (Fig. 3*B*, *left*). However, murine bone marrow-derived macrophages deficient in cathepsin B (Fig. 2*A*) or treated with CA-Me even at the highest dose (100 μM) (Fig. 3*B*, *right*) could not reach the inhibition levels achieved in THP-1 or human PBMC. In THP-1 cells, the inhibitory responses to CA-Me (Fig. 3*B*, *right*) were comparable to those observed in CA-Me-treated tumor cell invasion assays and intracellular cathepsin B inhibition analysis, which showed ~50–70% inhibition at a 10-μM concentration (21). Defects in TNF-α production in *Ctsb*$^{-/-}$- or CA-Me-treated macrophages were not due to lack of response to LPS because similar levels of TNF-α mRNA were induced by LPS in these cells (Fig. 2*B*), which is in line with previous studies showing that CA-Me had no effects on the expression of cytokine mRNAs induced by LPS (45). Incomplete inhibition of TNF-α production in *Ctsb*$^{-/-}$ or CA-Me-treated bone marrow-derived macrophages suggests that a cathepsin B-independent route can mediate TNF-α secretion at least in part in certain cell types. At this moment, the cathepsin B-independent secretion of TNF-α appears to be independent of calpain, cathepsin L, cathepsin G, and cathepsin K because their inhibitors failed to further prevent TNF-α secretion in *Ctsb*$^{-/-}$ macrophages (Fig. 3*B*, *right*). We further verified that the TNF-α-inhibitory effect of CA-Me was due to specific inhibition of cathepsin B because no such effect was detected in *Ctsb*$^{-/-}$ bone marrow-derived macrophages (Fig. 3*B*, *right*).

In summary, this study identified a new role for cathepsin B. We provided the first evidence that intracellular cathepsin B activity is involved in the trafficking of TNF-α-containing vesicle to the plasma membrane. Cathepsin B has been implicated in a number of inflammatory diseases and tumor progression and considered as a therapeutic target (46, 47). A novel role of cathepsin B in TNF-α-containing vesicle trafficking reveals new aspects of cathepsin B inhibitors for anti-inflammatory therapeutics and vesicle trafficking.

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Disclosures

The authors have no financial conflict of interest.

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