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RESEARCH ARTICLE

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Meprin β cleaves TREM2 and controls its phagocytic activity on macrophages

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Abstract

The triggering receptor expressed on myeloid cells 2 (TREM2) is a multifunctional surface protein that affects survival, migration, and phagocytic capacity of myeloid cells. Soluble TREM2 levels were found to be increased in early stages of sporadic and familial Alzheimer's disease (AD) probably reflecting a defensive microglial response to some initial brain damage. The disintegrin and metalloproteases (ADAM) 10 and 17 were identified as TREM2 sheddases. We demonstrate that meprin β is a direct TREM2 cleaving enzyme using ADAM10/17 deficient HEK293 cells. LC-MS/MS analysis of recombinant TREM2 incubated with meprin β revealed predominant cleavage between Arg136 and Asp137, distant to the site identified for ADAM10/17. We further demonstrate that the metalloprotease meprin β cleaves TREM2 on macrophages concomitant with decreased levels of soluble TREM2 in the serum of *Mep1b*$^{-/-}$ mice compared to WT controls. Isolated BMDMs from *Mep1b*$^{-/-}$ mice showed significantly increased full-length TREM2 levels and enhanced phagocytosis efficiency compared to WT cells. The diminished constitutive shedding of TREM2 on meprin β deficient macrophages could be rescued by ADAM stimulation through LPS treatment. Our data provide evidence that meprin β is a TREM2 sheddase on macrophages and suggest that multiple proteases may be involved in the generation of soluble TREM2.

Abbreviations: AD, Alzheimer's disease; ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; BMDM, bone marrow derived macrophage; CTF, C-terminal fragment; ICD, intracellular domain; TREM2, triggering receptor expressed on myeloid cells 2.

Dennis Kristopher Berner and Luisa Wessolowski contributed equally to this work.

KEYWORDS

ADAM10, Alzheimer's disease, cell surface protein, meprin β, metalloprotease, phagocytosis, protein-protein interaction, TREM2

1 | INTRODUCTION

Triggering receptor expressed on myeloid cells 2 (TREM2) is a type 1 transmembrane protein that is expressed on myeloid cells such as macrophages, dendritic cells, and microglia.[1] TREM2 is thought to be involved in different signaling pathways, thereby influencing cell differentiation, survival, proliferation, activation, and phagocytic potential.[2] Several mutations within the stalk region and the Ig-like V type domain of TREM2 were associated with an increased risk to develop Alzheimer's disease (AD).[3-6] If and how TREM2 function affects amyloid plaque metabolism is controversially discussed. It is reported that a loss of TREM2 function affects plaque morphology leading to larger and more diffuse plaques associated with more neuritic pathology and less clustered microglia (summarized in (7)). Along the same line, it was recently demonstrated that in the absence of functional TREM2 early amyloidogenesis is accelerated due to reduced phagocytic clearance of amyloid seeds despite reduced plaque-associated ApoE.[8] On the contrary, it was shown that TREM2 deficiency eliminates TREM2-positive inflammatory macrophages and ameliorates pathology in AD mouse models.[9] Although no definite endogenous ligand has been described to date a preference for anionic and lipid-like substances has been observed.[10-12] It was shown that TREM2 can bind LPS[13] and Aβ peptides,[14-16] which induces phagocytosis in cells. However, TREM2 cannot transduce the signal by itself, but requires the adaptor protein DNAX-activation protein 12 (DAP12), which can be phosphorylated, and then, stimulates a variety of intracellular pathways.[13,17] TREM2 is shed from the cell surface by the disintegrin and metalloproteases 10 and 17 (ADAM10 and ADAM17), leaving a C-terminal fragment (CTF) that is prone to proteolysis by the γ-secretase complex[18-21] (Figure 1A). Cleavage of TREM2 by ADAM10 occurs between His157 and Ser158, which is enhanced by the AD-associated variant H157Y.[20,21] Shedding of TREM2 from the cell surface may terminate signaling, and therefore, reduce the phagocytic capacity of microglia and macrophages.[18,20] The resulting soluble TREM2 (sTREM2) was shown to induce ERK and MAPK14 signaling in bone marrow derived macrophages (BMDM)[22] and NFκB signaling in microglia inducing pro-inflammatory cytokine expression and prolonged cell survival.[13,23] Additionally, sTREM2 increases early in AD and may serve as a surrogate marker for microglial activation.[18,24]

Meprin β belongs to the astacin family of metalloproteinases and exhibits striking cleavage specificity with a preference for negatively charged amino acids around the scissile bond.[25] Similar to ADAM10 and ADAM17, meprin β was found to cleave several cell surface proteins such as the amyloid precursor protein (APP) at the β-secretase site,[26] the IL-6 receptor on human granulocytes to induce IL-6 trans-signaling,[27] and CD99 on endothelial cells, thereby promoting transendothelial cell migration.[28,29]

Here, we demonstrate that meprin β sheds membrane bound TREM2 and rapidly degrades the soluble ectodomain. However, the identified major cleavage site for meprin β in TREM2 between Arg136 and Asp137 is N-terminal to the ADAM10 site, thus, generating slightly different CTFs and soluble TREM2 species. Meprin β co-expression with TREM2 significantly decreased the phagocytic potential of macrophages. Physiological relevance of this proteolytic cleavage is supported by strong accumulation of TREM2 on the surface of BMDMs isolated from meprin β knock-out mice and decreased serum levels of sTREM2 in these animals compared to wild-type controls.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transient transfection

Cells were cultured in DMEM (Dulbecco's modified Eagle's medium; Gibco), supplemented with 10% (v/v) of FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. Culture conditions were under a humidified atmosphere (5% of CO_2) at 37°C. Generation of HEK293_ADAM10/17$^{-/-}$ cells was previously described.[30] For transient transfection, cells were seeded in 10 cm cell culture dishes. Plasmid cDNA for hTREM2, meprin β, ADAM10, empty vector (pcDNA3.1), or the TREM2-DAP12 fusion construct[20] was mixed with polyethylenimine (1:3) (Sigma-Aldrich) in serum-free medium and incubated at room temperature for 30 minutes. After 24 hours of transfection, medium was changed to serum-free DMEM to avoid inhibition of meprin β through FCS. For CTF analysis, 5 μM of DAPT were added to the medium at 5 and 20 hours. Cells were harvested 24 hours posttransfection. In the relevant experiments, 50 nM each of recombinant soluble meprin β (smeprin β),[31] smeprin α,[32] or of sADAM10[33] were added to the serum-free DMEM.

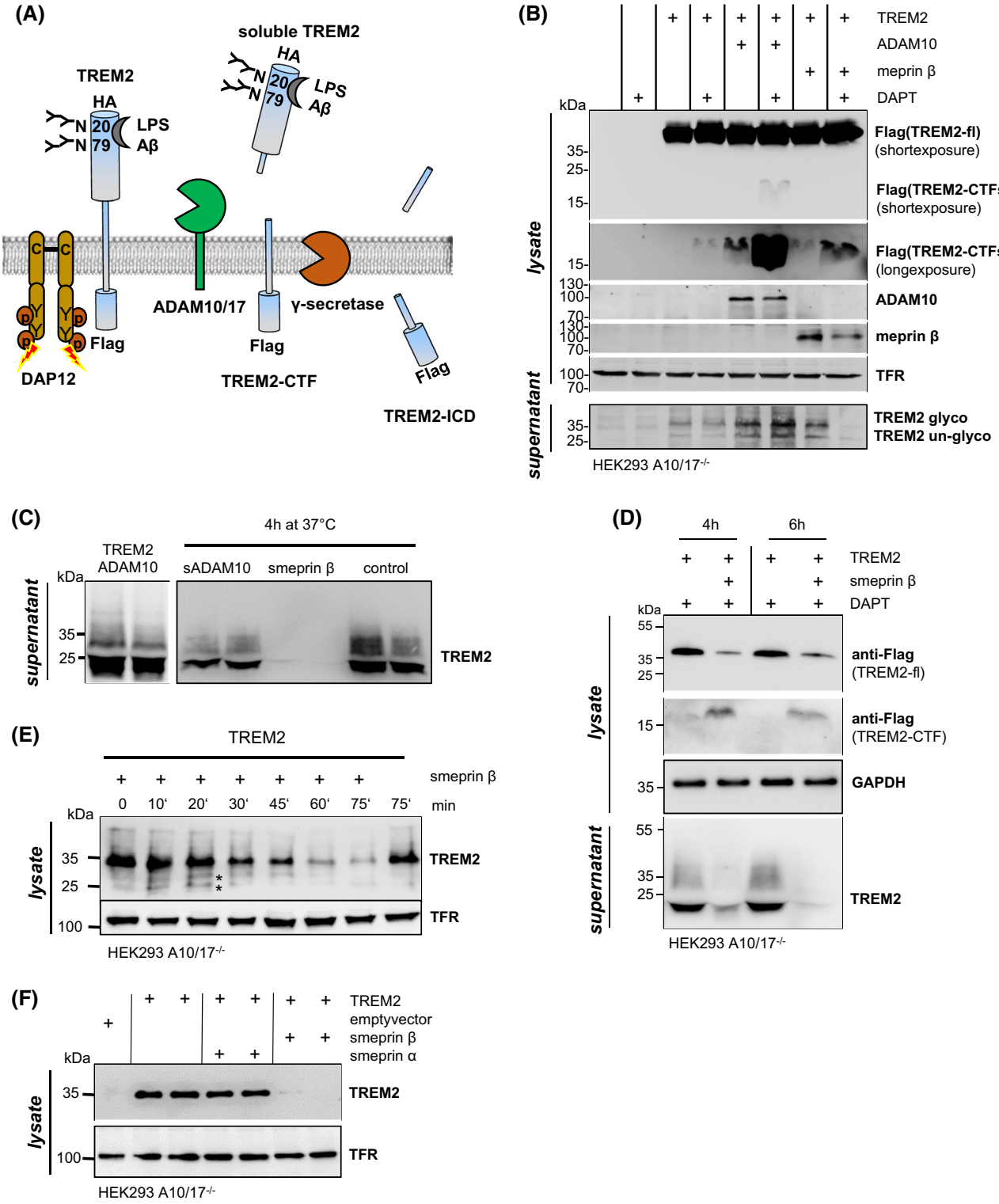

FIGURE 1 TREM2 is shed by meprin β in HEK293_ADAM10/17$^{-/-}$ cells. (A) Illustration of membrane bound TREM2 and DAP12. Upon shedding by ADAM10, the remaining C-terminal fragment (CTF) of TREM2 is cleaved within the membrane by γ-secretase, thereby releasing the intracellular domain (ICD). (B) Co-expression of TREM2 with meprin β or ADAM10 in HEK293_ADAM10/17$^{-/-}$ cells and Western blot analyses of TREM2 fragments in membrane-enriched fractions and cell supernatants. Anti-TFR antibody was used as loading control. (C) Conditioned media from TREM2 and ADAM10 transfected HEK293_ADAM10/17$^{-/-}$ cells were incubated with the recombinant ectodomains of meprin β and ADAM10 at 37°C for 4 hours. sTREM2 levels were analyzed by Western blot. (D) HEK293_ADAM10/17$^{-/-}$ cells transfected with TREM2 were incubated with recombinant meprin β and proteins from membrane-enriched fractions and cell supernatants were analyzed by Western blot. (E) Time series showing the cleavage of membrane bound TREM2 in HEK293_ADAM10/17$^{-/-}$ cells by recombinant soluble meprin β analyzed by Western blot. (F) Incubation of TREM2 overexpressing HEK293_ADAM10/17$^{-/-}$ cells with recombinant meprin α or meprin β and analysis of TREM2 by Western blot. All transient cell transfections and Western blot analyses were at least performed in biological triplicates

2.2 | Cell lysates, SDS-PAGE, and Western blot

Cell supernatants were collected and cells were harvested with a cell scraper and washed three times in ice-cold PBS. Cells were then incubated in membrane fractioning buffer (1 mM of EDTA, 1 mM of EGTA, 10 mM of TRIS, pH 7.0) for 30 minutes at 4°C and subsequently snap frozen in liquid nitrogen. After centrifugation for 30 minutes at 13 000 rpm at 4°C, pellets were suspended in EDTA-containing lysis buffer (1% (v/v) of Triton X-100 in PBS, pH 7.4) and incubated for 45 minutes. Lysates were centrifuged for 20 minutes at 15 000*g* at 4°C and protein amount was determined using the BCA protein assay kit (Thermo Fisher Scientific) following the manufacturer's instructions. Lysates were denatured in sample buffer including DTT for 10 minutes at 95°C.

Conditioned medium was collected after 24 hours, centrifuged at 1100*g* for 5 minutes and supplemented with a protease inhibitor cocktail (cOmplete EDTA-free, Roche). Supernatants were frozen at −20°C before analysis. For the analysis of soluble TREM2, 50 µL of Concanavalin A beads (C9017, Sigma) were added to 1 mL of supernatant and samples were incubated overnight. Beads were washed three times with sterile PBS before denaturation with sample buffer including DTT for 10 minutes at 95°C.

Samples were separated by SDS-PAGE (10% or 14% of gel) and blotted in a Tank Blot onto a PVDF-membrane. For immunoblot detection, the following antibodies were used: anti-human TREM2 (AF1828; R&D Systems; 1:1000), anti-murine TREM2 (clone 5F4)[34]; anti-flag (F1804; Sigma; 1:2000), anti-human meprin β (polyclonal, Pineda; 1:1000), anti-myc (9B11; Cell Signaling), anti-his (34660; Qiagen; 1:1000), anti-GAPDH (14C10; Cell Signaling; 1:2000), anti-TFR (ab84036; Abcam; 1:1000). The HRP-conjugated anti-goat (Jackson ImmunoResearch), anti-mouse (Jackson ImmunoResearch), and anti-rabbit (Jackson ImmunoResearch) were used as secondary antibodies.

2.3 | Bone marrow derived macrophages (BMDMS)

Macrophages derived from meprin β knock-out mice (*Mep1b*$^{-/-}$) were analyzed for TREM2 levels using murine-specific anti-TREM2 antibody (clone 5F4).[34] Mice were kept under specific pathogen free conditions in isolated ventilated cages, on a 12 hours light-dark cycle with food and water ad libitum. Mice were handled in accordance to the Guide for the Care and Use of Laboratory Animals of the German Animal Welfare Act on protection of animals. All animal protocols were approved by the Central Animal Facility of the University of Kiel and the relevant German authorities.

For isolation of primary macrophages, mice were killed by cervical dislocation. To get access to the BMDMs, extremities where amputated and transferred into sterile PBS. All surgical examinations took place under endotoxin-free conditions under a cell culture hood. Skin and muscles were carefully detached to isolate femur, crus, and humerus. Epiphysis were clipped off to allow access to the bone marrow in the medullary cavity of the diaphysis. By usage of sterile cannula (0.4 × 20 mm, Sterican, Braun) the medullary cavity was washed several times with DMEM. The obtained bone marrow in DMEM was then isolated with a cell strain (Falcon, A corning brand, 100 µm) and transferred into 50 mL Falcons. Collected cells where counted using a hemocytometer (C-chip, NanoEenTek, Neubauer improved, dhc-N01) and transferred into uncoated 10 cm cell culture dishes (TC-dish 100, Cell+, Sarstedt) in a density of 10^7 cells/dish and cultured in 10 mL DMEM (10% (v/v) of FCS) followed by substitution of 0.5 µg macrophage colony stimulating factor (M-CSF) per 10 mL DMEM. Cells were incubated for 5 days at 37°C. After 48 hours another 5 mL DMEM including 250 ng M-CSF were added. After 5 days, plates were washed three times with sterile PBS and macrophages were prepared for Western blotting or phagocytosis assay as described.

2.4 | Phagocytosis assay

Phagocytosis of fluorogenic *Escherichia coli* particles (pHrodo green *E coli*, Thermo Fisher Scientific) was analyzed using HEK293T ADAM10/17$^{-/-}$, PMA-induced THP-1, and U937 cells as well as primary macrophages (BMDMs) from mice. Cells were seeded in 96-well plates at a density of 8×10^4 cells per well 24 hours before phagocytosis measurements. After collection of the supernatants cells were washed two times with Live cell imaging solution (Thermo Fisher Scientific). *E coli* particles were added in a suspension of 1 µg/mL in Live cell imaging solution at a total of 50 µL/well. As a negative control, phagocytosis was inhibited with 10 µM of cytochalasin D (Life technologies), which was added 30 minutes prior to addition of pHrodo *E coli* bioparticles. Samples were incubated at 37°C for 120 minutes. Afterward, the suspension was carefully collected and cells were washed three times with MACS buffer (PBS pH 7.4, 0.5% (w/v) of BSA (Albumin Fraction V), 2 mM of EDTA). Cells were subsequently analyzed by either fluorescence-activated cell scanning (FACS) or fluorescence measurements with a microplate reader (Spark®, Tecan Group Ltd.). For Flow cytometric analysis of the phagocytosis assay, we used the FACSCanto (BD Biosciences). Data were analyzed with *FACSDiva* (BD Biosciences) and *FLOWJO Software* (Tree Star Inc).

2.5 | Deglycosylation of recombinant TREM2

Human recombinant TREM2 ectodomain (Sino Biological) was incubated with soluble recombinant meprin β in 20 mM of HEPES, pH 7.5 for 60 minutes at 37°C. For PNGaseF digestion, 4.5 µg of recombinant TREM2 were incubated with 5 nM of soluble meprin β in HEPES buffer for 2 hours. The samples were deglycosylated with PNGaseF kit (New England Biolabs) according to manufacturer's instructions and separated by SDS-PAGE. For Coomassie staining, gels were incubated in Coomassie Brilliant Blue R-250 Dye (Thermo Fisher Scientific) and subsequently destained.

2.6 | C-Terminal labeling and LC-MS/MS analysis

About 10 µg of human recombinant TREM2 (Sino Biological) were incubated with 5 nM soluble meprin β in HEPES buffer for 2 hours at 37°C. The samples were separated by SDS-PAGE and stained with Coomassie. To determine the C-terminus of the protein, a C-terminal labeling experiment was performed. Samples were excised from the gel band and further digested in heavy water ($H_2{}^{18}O$, 97% pure) with an additional protease (here LysargiNase and chymotrypsin). Newly formed C-termini, or neo-C-termini, will incorporate heavy water ($H_2{}^{18}O$), and can then be distinguished from the C-termini generated by the incubation of TREM2 with meprin β, which was performed using normal light water ($H_2{}^{16}O$).

Bands from the SDS-PAGE (Figure 2C) were excised, halved (split into two samples), cut into pieces of approximately 1 mm^3, destained, reduced with 10 mM of dithiothreitol at 56°C, alkylated with 55 mM of iodoacetamide at room temperature, washed with ammonium bicarbonate and acetonitrile (ACN), and dried in a vacuum centrifuge. Samples were subsequently digested overnight at 37°C in either 100 ng of chymotrypsin or LysargiNase (10 mM of ammonium bicarbonate, 2 mM of $CaCl_2$) in heavy water ($H_2{}^{18}O$,

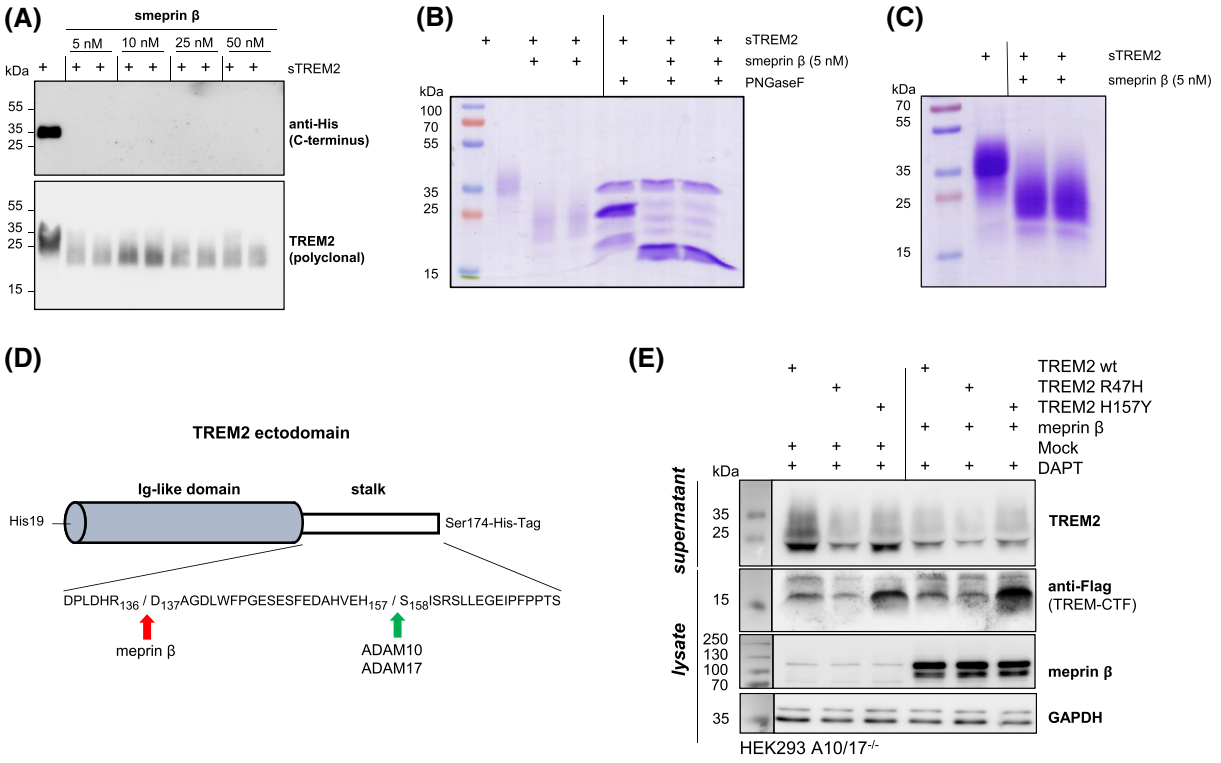

FIGURE 2 Meprin β cleaves TREM2 between arginine 136 and aspartate 137. (A) Incubation of sTREM2 with recombinant soluble meprin β (smeprin β) led to the generation of TREM2 fragments analyzed by Western blot (AF1828; R&D Systems; 1:1.000). (B) sTREM2 was incubated with smeprin β for 2 hours at 37°C and subsequently digested with PNGaseF. The meprin β generated TREM2 fragment is about 10 kDa smaller than the uncleaved TREM2 as analyzed by SDS-PAGE. (C) Coomassie stained gel of sTREM2 incubated with meprin β (2 hours at 37°C) that was used for determination of cleavage sites. Gel bands were excised, split into two samples and digested with either chymotrypsin or LysargiNase in heavy water. (E) Illustration of the TREM2 ectodomain construct used for cleavage site identification (sTREM2). While TREM2 is cleaved by ADAM10 and ADAM17 between histidine 157 and serine 158, the major meprin β cleavage site is further N-terminal between arginine 136 and aspartate 137. (F) Co-expression of TREM2 WT and AD-associated variants with meprin β in HEK293_ADAM10/17$^{-/-}$ cells and Western blot analyses of TREM2 fragments in membrane-enriched fractions and cell supernatants. Anti-GAPDH antibody was used as loading control. All transient cell transfections and Western blot analyses were at least performed in biological triplicates

97% pure). Following overnight digestion, peptides were extracted from the gel by subsequent sonication and shaking in 1% of formic acid (FA), 50% of ACN in 1% of FA, and 90% of ACN in 1% of FA, respectively. The samples were dried in a vacuum centrifuge and the peptides resuspended in 3% of ACN, 0.1% of TFA for further measurements with LC/MS.

In-gel digested samples were analyzed on a Dionex Ultimate 3000 nano-UHPLC coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific). The samples were washed on a trap column (Acclaim Pepmap 100 C18, 5 mm × 300 µm, 5 µm, 100 Å, Dionex) for 4 minutes with 3% of ACN/0.1% of TFA at a flow rate of 30 µL/min prior to peptide separation using an Acclaim PepMap 100 C18 analytical column (50 cm × 75 µm, 2 µm, 100 Å, Dionex). A flow rate of 300 nL/min using eluent A (0.05% of FA) and eluent B (80% of ACN/0.04% of FA) was used for gradient separation. Spray voltage applied on a metal-coated PicoTip emitter (10 µm tip size, New Objective, Woburn) was 1.6 kV, with a source temperature of 250°C. Full scan MS spectra were acquired between 300 and 2000 *m/z* at a resolution of 70 000 at *m/z* 400. The ten most intense precursors with charge states greater than 2+ were selected with an isolation window of 3.0 *m/z* and fragmented by HCD with normalized collision energies of 27 and at a resolution of 17 500. Lock mass (445.120025) and dynamic exclusion (15 seconds) were enabled.

The MS raw files were processed by Proteome Discover 2.2 (Thermo, version 2.2.0.388) and MS/MS spectra were searched using the Sequest HT algorithm against a database containing common contaminants (45 sequences) and the canonical human database (20 195 sequences). A semi-enzyme-specific search was performed for both chymotrypsin and LysargiNase with three and two missed cleavages allowed, respectively. An MS1 tolerance of 10 ppm and a MS2 tolerance of 0.02 Da was implemented. Oxidation (15.995 Da) of methionine residues was set as a variable modification along with C-terminal incorporation of ^{18}O (2.00425 Da). Carbamidomethyl (57.02146 Da) on cysteine residues was set as a static modification. Minimal peptide length was set to six amino acids and a peptide false discovery rate (FDR) was set to 1%.

2.7 | Generation of TBS soluble fractions

Snap-frozen brains were crunched on liquid nitrogen to generate brain powders. Several milligrams of brain powder were then thoroughly homogenized in 10 times the volume (w/v) of ice-cold TBS buffer (50 mM of Tris, 150 mM of NaCl, pH 7.4) by applying multiple strokes in protein low binding tubes (Eppendorf) using a syringe and a 26G needle. In the following, samples were incubated 30 minutes on ice with vortexing every 10 minutes. Upon centrifugation (10 minutes, 5000*g*) supernatants were subjected to ultracentrifugation (60 minutes, 130 000*g*). Supernatants were collected and frozen at −20°C until analysis.

2.8 | Murine TREM2 ELISA

For the quantification of sTREM2 levels in serum from wild-type and meprin β knock-out mice, we used the Meso Scale Discovery (MSD) platform employing the assay essentially as previously described.[35] We analyzed only male mice, as we have previously observed a gender-specific difference in TREM2 expression at a significantly lower level in female mice than in male littermates. First, an MSD Gold small spot streptavidin plate was coated with blocking buffer (3% of BSA, 0.05% of tween-20 in PBS, pH 7.4) overnight at 4C. On the next day, the plate was incubated for 90 minutes at RT with 0.125 µg/mL of biotinylated polyclonal goat anti-mouse TREM2 capture antibody (BAF1729; R&D Systems) diluted in blocking buffer. In the meantime, serum samples and TBS fractions were diluted 1:10 and 1:2, respectively, in sample dilution buffer (1% of BSA, 0.05% of tween-20 in PBS, pH 7.4) supplemented with protease inhibitor mix (Sigma). The plate was then washed two times with wash buffer (0.05% of tween-20 in PBS, pH 7.4) and serum samples were subsequently transferred onto the MSD plate for 120 minutes incubation at RT. The plate was washed two times with wash buffer and incubated in the following with 1 µg/mL rat monoclonal anti-mouse TREM2 detection antibody (clone 5F4, (34)) diluted in blocking buffer for 60 minutes at RT. The plate was again washed two times with wash buffer, and then, incubated with a SULFO-TAG-labeled goat anti-rat secondary antibody (1:1000; MSD) diluted in blocking buffer for 60 minutes at RT. Before the actual measurement, the plate was washed two times with wash buffer and another two times with PBS, pH 7.4. MSD read buffer was added to the plate and the light emission at 620 nm after electrochemical stimulation was measured using the MSD Sector Imager 2400. Concentrations of sTREM2 were finally calculated using the MSD Discovery Workbench software (v4.0.12). As a standard, we used recombinant murine TREM2 (Hölzel Diagnostika). For standard curve determination, two-fold serial dilutions were performed in sample dilution buffer supplemented with protease inhibitor mix (Sigma) on the plate spanning concentrations from 4000 to 62.5 pg/mL. sTREM2 concentrations as calculated for TBS soluble fractions were normalized to the total protein concentration as determined using the BCA assay (Interchim). In all incubation steps at RT, the plate was shaken at 300 rpm. All plate washings were performed using a plate washer (ELx405, BioTek).

2.9 | Statistics

All statistical analyses were carried out using GraphPad Prism version 6.01 for Windows (GraphPad Software, La Jolla California, USA). For multiple comparison, a One-way ANOVA followed by a Newman-Keuls multiple comparison test was performed. The comparison of two test groups was performed using an unpaired two-tailed t test. Statistical significance was assigned at the following p-values: * $P < .05$ and ** $P < .01$; **** $P < .0001$. All statistical analyses were carried out using at least three independent biological replicates and error bars are presented as standard error of the mean (SEM) or as standard deviation (SD).

3 | RESULTS

3.1 | Meprin β cleaves TREM2 at the cell surface

Several cell surface proteins were found to be similarly cleaved by ADAM10/17 and meprin β.[26,27] Therefore, we hypothesized that TREM2 may also be a shared substrate and investigated its shedding by meprin β. To avoid influence of the known TREM2 sheddases ADAM10 and ADAM17 (Figure 1A) in our experiments, we employed Crispr/Cas generated HEK293_ADAM10/17$^{-/-}$ cells.[30] We co-expressed TREM2 together with meprin β and, as a positive control, together with ADAM10. As described previously[18] ADAM10 generated a TREM2-CTF that could be enriched in membrane fractions by the addition of the γ-secretase inhibitor DAPT (Figure 1B). Indeed, co-expression with meprin β also resulted in the generation of TREM2-CTF (15 kDa), which was prone to γ-secretase cleavage as observed for the ADAM10 mediated CTF (Figure 1B). As ADAM10 cleaves TREM2 at a defined position within the stalk region[18,20] increased levels of sTREM2 could be precipitated from the cell supernatant after co-expression (Figure 1B). Surprisingly, in the presence of meprin β only faint signals were detected for sTREM2 after precipitation, even less pronounced than in TREM2 single transfected cells (Figure 1B). Therefore, we hypothesized that meprin β might not only act as a sheddase of TREM2, but also further degrades the entire ectodomain of the receptor. As a control experiment, we generated sTREM2 through co-expression of TREM2 with ADAM10, and then, added the recombinant, active ectodomains of ADAM10 (sADAM10)[33] or meprin β (smeprin β)[31] to the conditioned medium (Figure 1C). While sADAM10 showed almost no effect on sTREM2, incubation with smeprin β resulted in complete degradation of the sTREM2 ectodomain (Figure 1C). To investigate whether membrane bound TREM2 is also a substrate of soluble meprin β, we transfected the HEK293_ADAM10/17$^{-/-}$ cells with TREM2 and incubated them with smeprin β. This resulted in a decrease of full-length TREM2 and an increase in CTFs (Figure 1D). Again, we found less sTREM2 in the supernatant of cells incubated with smeprin β compared to untreated cells. To gain further insight into the cleavage dynamics and to investigate if the complete degradation of TREM2 by meprin β can already occur on the cell surface, we performed a time course experiment. To do so, HEK293_ADAM10/17$^{-/-}$ cells were transfected with TREM2 and incubated with smeprin β for different times (Figure 1E). We observed that almost 50% of full-length TREM2 was already cleaved after 30 minutes. The polyclonal antibody raised against the ectodomain of human TREM2 revealed additional bands (Figure 1E, indicated by asterisks) at lower molecular weight after 10 and 20 minutes incubation that vanished later on, supporting the observation of TREM2 degradation at the cell surface. To further validate specificity of meprin β as a sheddase and degrading enzyme of TREM2, we additionally assessed TREM2 cleavage by meprin α, a close relative of meprin β.[36] However, meprin α showed no proteolytic activity against TREM2 (Figure 1F).

3.2 | TREM2 is cleaved by meprin β between arginine 136 and aspartate 137

To determine the meprin β cleavage site in TREM2, we used recombinant human TREM2 ectodomain comprising the whole stalk region and Ig-like domain (His19 to Ser174), fused with a His-tag at its C-terminus. In Western blot analysis, we found that incubation with 5 nM of meprin β is sufficient to cleave soluble TREM2 and generate a rather stable fragment (Figure 2A). TREM2 is highly N-glycosylated (Asn20 and Asn79) and it is difficult to determine the exact size of the cleavage fragments. Therefore, we used PNGaseF to deglycosylate TREM2. This experiment showed that the meprin β generated TREM2 fragment is about 10 kDa smaller than the uncleaved TREM2 (Figure 2B). A C-terminal ^{18}O-labeling strategy followed by LC-MS/MS analysis was employed for the identification of meprin β cleavage sites in glycosylated TREM2 (Figure 2C, Tables 1 and 2). One prominent cleavage site (in terms of the number of PSMs (Peptide Spectrum Matches)) between Arg136 and Asp137 in two separate experiments (Tables 1 and 2) could be identified. Besides this major cleavage site, we also found several minor cleavage events N- and C-terminal of acidic amino acid residues from positions 131 to 147. In summary, meprin β cleaves TREM2 within the stalk region predominantly between Arg136 and Asp137, which is distinct to the ADAM10/17 identified site between His157 and Ser158 (Figure 2D).[20,21,37] However, we additionally analyzed shedding of the AD-associated variant TREM2 H157Y by meprin β (Figure 2E). Interestingly, we observed that cleavage of TREM2 H157Y by meprin β resulted in increased TREM2-CTF levels compared to the

Annotated sequence	Position in protein	# PSMs	Theo. MH+ [Da]
[L].RKVLVEVLADPLDHRDAG.[D]	[122-139]	1	2003.10325
[L].RKVLVEVLADPLDHRDA.[G]	[122-138]	2	1946.08178
[L].RKVLVEVLADPLDHR.[D]	[122-136]	8	1760.01773
[L].RKVLVEVLADPL.[D]	[122-133]	1	1351.83076
[R].KVLVEVLADPLDHR.[D]	[123-136]	2	1603.91662

TABLE 1 C-terminal peptides that were identified for meprin β hydrolyzed TREM2 following an in-gel digestion with LysargiNase. Several cleavage sites were identified including 133L.134D, 136R.137D, 138A.139G, and 139G.140D. The most abundant meprin β cleavage site (in terms of the number of PSMs (Peptide Spectrum Matches)) was observed at 136R.137D

Annotated sequence	Position in protein	# PSMs	Theo. MH+ [Da]
[L].HGSEADTLRKVLVEVLADPL.[D]	[114-133]	1	2162.18156
[L].RKVLVEVLADPLDHRDA.[G]	[122-138]	4	1946.08178
[L].RKVLVEVLADPLDHR.[D]	[122-136]	51	1760.01773
[L].RKVLVEVLADPLDH.[R]	[122-135]	2	1603.91662
[L].RKVLVEVLADPLD.[H]	[122-134]	1	1466.85771
[L].RKVLVEVLADPL.[D]	[122-133]	3	1351.83076
[L].RKVLVEVLAD.[P]	[122-131]	2	1141.69393
[L].VEVLADPLDHRDAG.[D]	[126-139]	1	1506.7547
[L].VEVLADPLDHR.[D]	[126-136]	6	1263.66918
[L].VEVLADPLDH.[R]	[126-135]	1	1107.56807
[L].DHRDAGDLWFPGES.[E]	[134-147]	2	1601.69791
[L].DHRDAGDLWFPGE.[S]	[134-146]	1	1514.66588
[L].DHRDAGDLWFPG.[E]	[134-145]	1	1385.62329

TABLE 2 C-terminal peptides that were identified for meprin β hydrolyzed TREM2 following an in-gel digestion with chymotrypsin. Several cleavage sites were identified. The most abundant meprin β cleavage site (in terms of PSMs) was observed at 136R.137D

wild-type form, although the aa exchange at position 157 is not directly influencing the newly identified meprin β cleavage site at Asp137. Therefore, we assume that this variant is somehow more accessible to meprin β (localization, protein stability, etc) rather than triggering the cleavage site.

3.3 | Co-expression of meprin β and TREM2 decreases the phagocytic potential of cells

To assess the functional consequence of TREM2 degradation by meprin β, we performed a phagocytosis assay in different cell lines. To test the phagocytic potential in ADAM10/17 deficient HEK cells, a fusion construct of TREM2 and DAP12 was used as these cells do not naturally express DAP12[18] (Figure 3A). To test if this construct is proteolytically processed, it was co-expressed with ADAM10 or meprin β (Figure 3B). Isolated membrane fractions revealed a decreased signal for full-length TREM2 upon co-expression with both proteases, confirming cleavage of this TREM2-DAP12 construct. As expected, increased levels of sTREM2 were found in precipitated supernatants upon ADAM10 co-transfection and decreased levels upon meprin β co-expression (Figure 3B). The phagocytic potential of the cells was measured by the internalization of fluorogenic pHrodo beads.[18,20] These are *E coli* bioparticles conjugated to a dye emitting fluorescence in acidic milieu. Thus, a fluorescent signal can only be measured after endocytosis of the particles and transport to the lysosome. We observed that co-expression of TREM2 and meprin β decreases the phagocytic capacity of these cells significantly when compared to TREM2 single transfected cells (Figure 3C). A similar decrease in phagocytosis was detected after co-transfection of TREM2 and ADAM10 (Figure 3C).[18] To assess this in a more physiologically relevant cell type, we repeated this experiment using the human macrophage cell lines THP-1 and U937, which endogenously expresses DAP12. Therefore, cells were transfected with TREM2 alone or together with meprin β (which is barely detectable endogenously), and at the same time, were stimulated with PMA to induce a macrophage-like differentiation.[38] After 72 hours, pHrodo beads were added and the amount of phagocytosed beads was analyzed after 120 minutes. As seen in the HEK cell experiment, co-expression of meprin β with TREM2 reduced the phagocytic potential significantly (Figure 3D,E) in both macrophage cell lines.

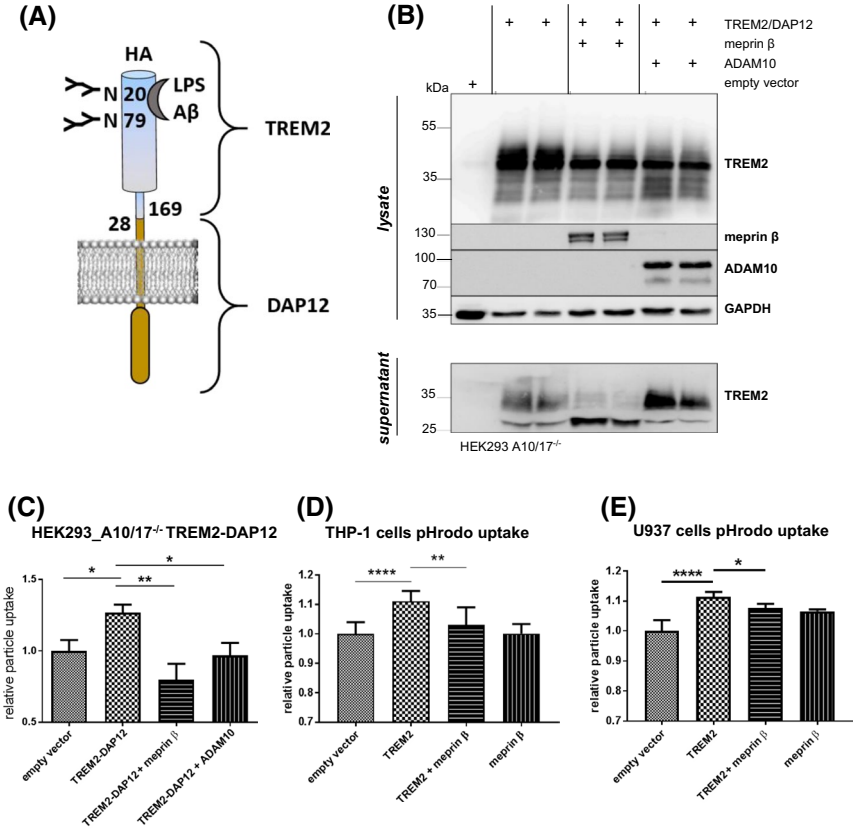

FIGURE 3 Impaired phagocytosis of fluorogenic *E coli* particles in cells co-expressing meprin β and TREM2. (A) Illustration of the TREM2/DAP12 fusion construct used in the phagocytosis assays. (B) Proteolytic cleavage of the overexpressed fusion constructs in HEK293_ADAM10/17$^{-/-}$ cells transfected with meprin β or ADAM10. Proteins from cell supernatants and membrane-enriched fractions were analyzed by Western blot. (C) Phagocytosis assay with transfected HEK293_ADAM10/17$^{-/-}$ cells. Relative particle uptake analyzed by FACS is indicated for differentially transfected cells. Data are represented as means of median fluorescence intensity ± SEM from two independent experiments and expressed relative to empty vector transfected control (n = 8 and 9, respectively). (D) Relative particle uptake of THP-1 cells transfected with wild-type TREM2 and meprin β (n = 10) was analyzed using a Tecan fluorescent reader measuring mean fluorescence intensity of a defined number of cells. (E) Relative particle uptake of U937 cells transfected with wild-type TREM2 and meprin β (n = 6) was analyzed using a Tecan fluorescent reader measuring mean fluorescence intensity of a defined number of cells. All transient cell transfections and Western blot analyses were at least performed in biological triplicates. Statistical differences were calculated by One-way ANOVA followed by a Newman-Keuls multiple comparison test. Comparison of two test groups was performed using an unpaired two-tailed *t* test (*$P < .05$; **$P < .01$; ****$P < .0001$)

3.4 | Meprin β controls TREM2 levels on primary macrophages

As we could show that membrane bound and soluble meprin β degrade TREM2, which leads to reduced phagocytosis, we were keen to evaluate if cleavage of TREM2 by meprin β is physiologically relevant. Meprin β has been reported to be expressed on macrophages important for cell migration and pro-inflammatory stimuli.[39,40] Thus, we isolated BMDMs from meprin β knock-out[41] and wild-type mice and analyzed the TREM2 levels. Mature TREM2 significantly accumulated in membrane-enriched fractions of macrophages deficient for meprin β (Figure 4A,B). After activating the BMDMs with LPS for 6 hours to induce ADAM mediated shedding, the signal intensity for mature TREM2 was similar to the one obtained for wild-type BMDMs (Figure 4A). This indicates a constitutive function for meprin β and an inducible shedding activity for ADAMs in TREM2 proteolysis. To assess a possible functional consequence, we performed the phagocytosis assay with these cells (non LPS stimulated) and found a significant increase in phagocytic potential for cells deficient for meprin β when compared to wild-type cells (Figure 4C). As a negative control, we added cytochalasin D (CytoD), an actin polymerization inhibitor that blocks phagocytosis (Figure 4C).[18] To further investigate, if meprin β is a constitutive sheddase of TREM2 in vivo, we analyzed the levels of soluble TREM2 in serum of *Mep1b*$^{-/-}$ mice. Indeed, we detected significantly reduced amounts of the soluble receptor in serum of *Mep1b*$^{-/-}$ animals compared to WT mice (Figure 4D).

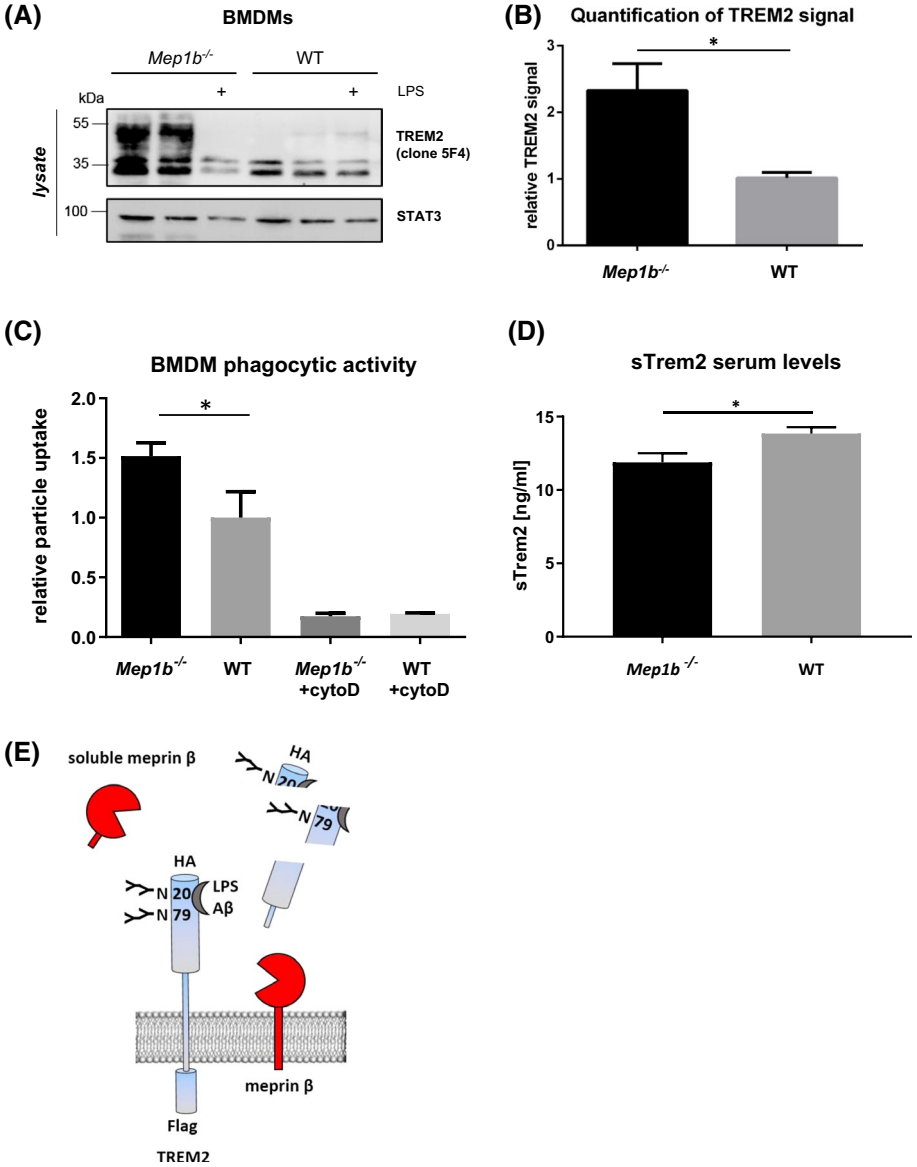

FIGURE 4 Cleavage of TREM2 on macrophages by meprin β. (A) Membrane-enriched fractions from BMDMs of *Mep1b*$^{-/-}$ mice showed higher levels of mature TREM2 than the wild-type controls visualized by Western blot. Enhanced levels of mature TREM2 at 45 kDa were detected (murine-specific anti-TREM2, clone 5F4) in samples of *Mep1b*$^{-/-}$ mice, which was decreased upon LPS stimulation. Anti-STAT3 was used as loading control. (B) Densitometric analysis of TREM2 band intensities in non LPS stimulated BMDMs from *Mep1b*$^{-/-}$ and wild-type mice as seen in (A). The comparison of two test groups (n = 5) was performed using an unpaired two-tailed *t* test. (*$P < .05$). (C) Phagocytosis assay with BMDMs from wild-type and *Mep1b*$^{-/-}$ mice. Cytochalasin D (CytoD) was used to block endocytosis. Relative particle uptake measured by FACS is indicated for different cells. Data are represented as means of median fluorescence intensity ± SD (n = 3). Statistical differences were calculated by One-way ANOVA followed by a Newman-Keuls multiple comparison test (*$P < .05$). (D) Determination of sTREM2 levels in serum reveals significantly lower levels in *Mep1b*$^{-/-}$ (n = 7, all male) than in wild-type mice (n = 5, all male). Data are represented as means ± SEM. (E) Cartoon illustrating the interaction of TREM2 with soluble and membrane bound meprin β resulting in the degradation of the TREM2 ectodomain

4 | DISCUSSION

Recently, Crowther and colleagues showed that on macrophages turnover of TREM2 is remarkably rapid, having a half-life of <1 hour. Notably, inhibition experiments revealed only minor contribution of ADAM10 in these cells.[21] The metalloprotease meprin β can cleave several cell surface proteins similar to ADAM10/17.[26,27] Hence, we investigated if TREM2 may also be a shared substrate and analyzed its shedding by meprin β. Indeed, TREM2 was cleaved by meprin β, and the soluble ectodomain of the receptor was even further degraded by the protease, which is a striking difference compared to ADAM10 cleavage. Meprin β can be physiologically shed from the cell surface by ADAM10 and

ADAM17, generating the soluble meprin β ectodomain that is still able to cleave substrates,[42-44] which was also confirmed for the cleavage of TREM2 (Figure 4E).

Besides ADAM10/17, we further validated the specificity of meprin β as a sheddase and degrading enzyme of TREM2. Therefore, we investigated TREM2 cleavage by the metalloprotease meprin α, which is closely related to meprin β.[36] Of note, meprin α displays a similar cleavage specificity with a preference for negatively charged amino acids in P1′ position of the cleavage site.[25] However, meprin α showed no proteolytic activity against TREM2. This observation is similar to APP cleavage, where meprin β but not meprin α can act as β-secretase.[45]

For meprin β the major cleavage site in TREM2 was identified between Arg136 and Asp137, which is N-terminal to the ADAM10/17 site. This cleavage site perfectly matches the meprin β preference for negatively charged amino acids at the P1′position.[25] Besides this major cleavage site, we also found several minor cleavage events N- and C-terminal of acidic amino acid residues from positions 131 to 147. Of note, for frontotemporal dementia (FTD) one missense mutation of TREM2 (D134G) is reported [46] that is in close proximity to the major meprin β cleavage site and may affect TREM2 shedding under certain conditions.

It was shown previously that the amount of functional TREM2 directly correlates with the phagocytic potential of cells.[18] This is further supported by disease-associated TREM2 variants found in AD, Nasu-Hakola disease (NHD), and FTD-like syndrome that do not reach the cell surface and dampen phagocytosis.[18,20] We were interested, if the activity of meprin β toward TREM2 alters the phagocytic potential of cells. Since meprin β has been reported to be expressed on macrophages, being important for cell migration and pro-inflammatory stimuli,[39,40] we analyzed BMDMs from *Mep1b*$^{-/-}$ mice and observed increased phagocytosis compared to wild-type cells. These findings indicate a correlation of meprin β levels and the amount of TREM2 on the cell surface of macrophages and consequently link meprin β levels to the phagocytic potential of these cells in vivo. However, we cannot fully exclude that additional meprin β substrates could influence phagocytosis or that the interaction of meprin β and TREM2 may have impact on other biological functions besides phagocytosis, such as migration. The physiological relevance of meprin β mediated TREM2 cleavage is further supported by significantly reduced levels of soluble TREM2 in the serum of *Mep1b*$^{-/-}$ mice compared to control animals. However, the role of meprin β in inflammatory diseases is still a controversial issue. Meprin β has been linked to inflammatory bowel disease[47] and it was shown to shed the IL-6R and to induce pro-inflammatory trans-signaling.[27] Meprin β promotes transendothelial migration of cells through CD99 cleavage, an important step for immune cells to reach the site of inflammation.[28] Additionally, meprin β can cleave, and thereby inactivate IL-6[48] and the important chemokine CCL2/MCP-1 leading to a reduced monocyte attraction.[49]

Taken together, we found that meprin β sheds and degrades TREM2 at the cell surface and that it controls the level of TREM2 on macrophages but not on microglial cells, at least under unchallenged conditions. We determined a major cleavage site for meprin β between Arg136 and Asp137, which is 21 aa N-terminal of the described ADAM10 cleave site. Additionally, we could show that meprin β can control the cell autonomous function of TREM2 and decreases the phagocytic potential of cells. Of note, this is not necessarily restricted to meprin β expressing cells, as we could show that the protease can act as membrane bound or soluble protease in cis and trans, respectively. In sum, soluble TREM2 generated by meprin β exhibits different properties compared to the ADAM-released ectodomain, which might interfere with already established approaches for the detection of sTREM2 in blood or CSF. This should be taken into account in TREM2 associated pathologies.

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CONFLICT OF INTEREST

C.H. collaborates with Denali Therapeutics, participated on one advisory board meeting of Biogen, and received a speaker honorarium from Novartis and Roche. C.H. is chief advisor of ISAR Bioscience.

AUTHOR CONTRIBUTIONS

DKB, LW, PA, and CBP conceived the study. DKB, LW, JS, KS, TK, FS, FA, AT, CH, PA, and CBP performed or designed experiments. KS, RL, GK, and CH provided material. DKB, LW, and CBP wrote the manuscript.

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