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Novel transmembrane protein 126A (TMEM126A) Couples with CD137L Reverse Signals in Myeloid cells

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Abstract

Members of the TNF family can promote signals in myeloid cells and both positively and negatively regulate the production of pro-inflammatory cytokines depending on the target myeloid cell type. Using the yeast-two hybrid system, we identified transmembrane protein 126A (TMEM126A) as a binding partner for CD137L (4-1BB ligand). We found that TMEM126A associated and co-localized with CD137L in a mouse macrophage cell line and knockdown of TMEM126A with siRNA abolished CD137L-induced tyrosine phosphorylation as well as up-regulation of M-CSF, IL-1β and TN-C expression. Knockdown of TMEM126A also blocked down-regulation of IL-1β and IL-6 expression induced by CD137L in thioglycollate-elicited primary peritoneal macrophages. Knockdown of TMEM126A by stable retroviral TMEM126A shRNA transduction also abolished CD137L-induced tyrosine phosphorylation and cell adherence. These findings identify a novel molecule that bridges TNF family cytokines and pro-inflammatory cytokine secretion in myeloid cells.

Keywords

CD137L; innate immunity; myeloid cells; reverse signals; TMEM126A

1. Introduction

Members of the TNF receptor superfamily are well known for regulating activities of T and B lymphocytes by recruiting TRAF (TNF receptor associated factor) adapter proteins that link to downstream kinases, such as IKK beta. Much less appreciated is that members of the TNF family when expressed on the surface of myeloid antigen-presenting cells can also transduce signals and these can modify the production of pro-inflammatory cytokines and additionally contribute to adaptive immunity [1]. However, the nature of the TNF family

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signals and how they are transmitted is not well understood. General intracellular mediators of these reverse signals include NF-kappa B, PI-3-kinase/Akt, and MAP kinases, but because of the short cytoplasmic domains of the TNF family molecules it is unclear how these pathways are recruited [2–4].

CD137 (4-1BB), a member of the tumor necrosis factor receptor (TNFR) family, is a co-stimulatory protein expressed on activated T cells, and its interaction with CD137 ligand (CD137L, 4-1BBL) that is generally expressed on APCs can regulate both positively and negatively many types of immune response [5–8]. Bi-directional signal transduction by CD137L/CD137 has received attention because ligation of CD137L can provide reverse signals for immune homeostasis [9]. CD137L-mediated reverse signals produce cellular responses in a variety of cell types including both myeloid and lymphoid immune cells [10]. We reported that cross-linking of CD137L with recombinant CD137-Fc protein (rCD137-Fc) enhanced adherence of bone marrow-derived macrophages, and increased the expression of ICAM-1, IL-1β, IL-6 and M-CSF [11]. Also, in thioglycollate-elicited peritoneal macrophages, CD137L ligation opposed the expression of IL-1β and IL-6 [12] and in myeloid progenitors CD137L signals suppressed differentiation into the dendritic cell lineage [9]. This suggests that CD137L reverse signals evoke positive or negative effects on innate immune responses depending on the extent of activation or differentiation state of the target cell type.

CD137L signal transduction pathways have been studied in both mouse and human cells [6]. In human monocytes, ligation of CD137L activates Src tyrosine kinase, p38, ERK1/2 and PI3K, and induces nuclear translocation of NF-κB [13]. In mouse monocytic cells, it leads to phosphorylation of a Src family tyrosine kinase and activates both Akt and mTOR/p70S6 kinase in parallel [11]. Since the signaling molecules associated with the cytoplasmic domain of CD137L remained to be identified, we studied the proximal mechanism by which cross-linking of CD137L produces cellular responses in murine macrophages. We identified a novel protein, TMEM126A, as a binding partner of CD137L, and demonstrated that the association of TMEM126A with CD137L is critical for CD137L reverse signaling. No reports exist of TMEM126A expression or function in the lymphoid system. Therefore, these data provide a novel molecular mediator of signaling in myeloid cell types.

2. Materials and Methods

2.1. Mice, reagents, and antibodies

Male C57BL/6 mice were obtained from Harlan (Indianapolis, IN) and maintained under specific pathogen-free conditions. Kyung Hee University Animal Care and Use Committee approved the handling of animals and experiment protocols. Recombinant mouse CD137 conjugated with human IgG-Fc (rCD137-Fc) was obtained from Adipogen (Seoul, Korea). Human IgG-Fc (hIgG-Fc) was from Accurate Chemical and Scientific Corp (Westbury, NY). Anti-CD137L mAb (TKS-1) and isotype rat IgG for cross-linking CD137L were purchased from R&D Systems (Minneapolis, MN). Anti-Flag Ab, anti-Myc Ab and PE- and FITC-conjugated secondary Abs, as well as isotype control antibody, were purchased from BD Bioscience (San Diego, CA). Anti-TMEM126A Ab (Y-12), secondary Abs for Western blotting, and RIPA lysis buffer, were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-CD137L pAb for Western blotting and anti-CD137L mAb–biotin (TKS-1) for immunofluorescence staining were supplied by R&D Systems (Minneapolis, MN), and anti-CD137L mAb for immunoprecipitation by Lifespan Biosciences (Seattle, WA). Streptavidin, Alexa Flor 555 and Alexa Flor 488 anti-rabbit IgG were purchased from Invitrogen (Carlsbad, CA) and anti-pTyr mAb (4G10 platinum) from Millipore (Bedford, MA). Rabbit anti-mouseTMEM126A-N pAb was prepared using the peptide derived from the N-terminal region (1–14 amino acid) of TMEM126A (Peptron, Daejeon, Korea)

2.2. Yeast two hybrid system

The yeast Gal-4 two hybrid system was used to screen protein(s) that might interact with mouse CD137L, following the protocol provided by Clontech (Invitrogen, Carlsbad, CA). In brief, the full length cDNA of CD137L was cloned in pGBK-T7, and confirmed by sequencing and protein expression in yeast-AH109. The transgenic pGBK-T7 was transformed with a mouse thymus cDNA library and plated on selection medium lacking tryptophan, leucine and histidine, followed by X-alpha gal assays. The inserts in positive clones were characterized and moue TMEM126A was identified four times.

2.3. Over-expression of Flag-tagged CD137L and Myc-tagged TMEM126A in HEK 293 cells

Mouse cDNAs for CD137L-full length (F), -N terminal (N), -C terminal (C) and TMEM126A were amplified by PCR using pairs of gene specific primers with EcoRI sites at their 5' ends and XhoI sites at their 3' ends. The PCR products were digested with the two restriction enzymes and cloned into pCS4-3xFlag and pCS4-3xMyc, respectively [14]. The DNA sequences and protein expression in HEK 293 cells were confirmed.

2.4. Preparation of thioglycollate-elicited mouse peritoneal macrophages

In brief, mice were i.p. injected with 3.8% thioglycollate solution in DPBS (Gibco, Carlsbad, CA) for 3 days. Five ml of DPBS was then i.p. injected for lavage, and the peritoneal lavage fluid was centrifuged at 1,000 rpm for 5 min to collect cells. The pellets were washed 3 times with DPBS and re-suspended in RPMI/10% FBS. Routinely > 90% of the isolated cells were $CD11b^+$ cells, as determined by flow cytometric analysis.

2.5. Cell stimulation

To cross-link CD137L on cells, we exposed the cells to plate-bound rCD137-Fc or anti-CD137L mAb (TKS-1). To immobilize rCD137-Fc, TKS-1, control hIgG-Fc or isotype control rat IgG on culture plates, hIgG-Fc and rCD137-Fc or rat IgG and TKS-1 were incubated in 12-well plates at 37°C for 1 h in a CO_2 incubator. The wells were rinsed twice with DPBS, incubated with RPMI/10 % FBS at 37°C for 1 h in the CO_2 incubator and again rinsed twice with DPBS. RAW264.7 cells or thioglycollate-elicited peritoneal macrophages were then incubated on the coated plates for the indicated times.

2.6. Cell adherence assay

Retroviral scrambled or TMEM126A shRNA transduced-RAW264.7 cells were plated at 1×10^5 cells/well in 96-well, flat-bottom plates coated with hIgG-Fc or rCD137-Fc and Rat IgG or TKS-1. At 1 hr after incubation, cells were washed 5 times with DPBS to remove non-adhesion cells and then, the number of adhesive cells was determined using a CellTiter 96s Non-Radioactive Cell Proliferation Assay kit (Promega, Madison, WI, USA).

2.7. Immunopreciptation and pull-down assays

Cellular proteins were extracted with RIPA lysis buffer in the presence of phosphatase inhibitors and protease inhibitors, immunoprecipitated with isotype IgG or the appropriate Abs, and pulled-down with hIgG-Fc or rCD137-Fc for 3 h at 4°C. Dynabead protein G (Invitrogen, Carlsbad, CA) was added to form Dynabead protein G-bound immunoprecipitates or pull-down complexes. After incubation for 16 h at 4°C, they were washed, fractionated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, Bedford, MA). Proteins of interest were detected with primary Abs and secondary Ab-HRP, and bound Ab was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Little Chalfont, England).

2.8. Immunofluorescence staining

Staining was performed at room temperature. RAW264.7 cells or transfected HEK 293 cells (3×10^5 cells) were plated on poly-L-lysine-coated glass slides (BD Bioscience, San Diego, CA), washed with PBS, fixed in 3.7% paraformaldehyde for 15 min, quenched with 50 mM ammonium chloride for 10 min, permeabilized with 0.5% Triton X-100 for 5 min, blocked with 0.2% gelatin in PBS for 30 min, and stained with the appropriate primary Abs for 30 min. After three washes with 0.2% gelatin in PBS, the cells were stained with secondary Ab for 30 min, and washed three times with 0.2% gelatin in PBS. Cover slips were applied with FluorSave mounting solution (Merck, Darmstadt, Germany), and the cells were visualized with an immunofluoresence microscope. To minimize channel overlap of the dual colored samples, we used a sequential scanning technique that excited one dye at a time.

2.9. Western blotting

RAW264.7 cells were cultured, and proteins were extracted with RIPA lysis buffer in the presence of phosphatase inhibitors and protease inhibitors. Equal amounts of protein were diluted with 4 × SDS sample buffer, applied to SDS-PAGE gels, fractionated, and transferred to nitrocellulose membranes. Proteins of interest were detected with primary Abs and secondary Ab-HRP, and bound Ab was detected by enhanced chemiluminescence.

2.10. Flow cytometry

Cells were collected and washed with cytometric buffer (PBS containing 1% BSA and 0.1% sodium azide), incubated with anti-FcγR mAb (2.4G2) for 5 min on ice, and stained with Abs for 30 min on ice. After washing with cytometric buffer, samples were analyzed with a Cell Lab Quanta SC (Beckman Coulter, Miami, FL)

2.11. siRNA transfection

RAW264.7 cells (5×10^4 cells) or peritoneal macrophages (5×10^5 cells) were plated in 12-well plates coated with 100 ng/ml hIgG-Fc or rCD137-Fc. Twenty nM siRNA duplex was diluted and incubated for 5 min in 100 μl Opti-MEM I Reduced Serum Medium (Gibco, Carlsbad, CA). An appropriate volume of Lipofectamine RNAiMAX (Invitrogen, Carlsbad, CA) was diluted in 100 μl Opti-MEM I Reduced Serum Medium and also incubated for 5 min. The diluted siRNA and Lipofectamine RNAiMAX were mixed and incubated for 20 min. The siRNA duplex-liposome complexes were then added to the plated cells and incubation continued for the indicated times. After 1 or 2 days, the cells were lysed, and RNA and protein was extracted for RT-PCR and Western blotting, respectively. The siRNA duplex pairs (Bioneer, Daejeon, Korea) were as follows: GCU GCC UUC AUU CUG CCG A (TMEM126A-sense), UCG GCA GAA UGA AGG CAG C (TMEM126A-antisense), CCU ACG CCA CCA AUU UCG U (scrambled-sense), ACG AAA UUG GUG GCG UAG G (scrambled-antisense)

2.12. Establishment of TMEM126A-deficient mouse macrophage cell line

Stable introduction of TMEM126A-specific shRNA into RAW264.7 cells via retroviral infection was done as described in the protocol provided by Origene (Rockville, MD). In brief, shRNA constructs against mouse TMEM126A and scrumbled negative control non-effective shRNA cassette in pGFP-V-RS vector (Origene, Rockville, MD) were transiently transfected into Plat-E cells packaging shRNA retrovirus. shRNA retrovirus were then transduced into RAW264.7 cells by retroviral infection. Stable transduced cells were selected by incubating with 1.0 μg/ml puromycin. TMEM126A shRNA function was determined by Western blotting. The shRNA duplex pairs were as follows: AGU CAU AAA CCA AGU ACU AGC AAA GAU GA (TMEM126A-sense), UCA UCU UUG CUA GUA CUU GGU UUA UGA CU (TMEM126A-antisense)

2.13. Quantitative analysis of mRNA expression

Real-time PCR analysis (MiniOpticon, Bio Rad, USA) was performed using PCR Master Mix (Power SYBR Green; Applied Biosystems) to quantify expression of TMEM126A, IL-1β, IL-6, TN-C and M-CSF (normalized to GAPDH expression). The primer pairs were as follows: TTG TCA AGC TCA TTT CCT GGT (GAPDH-forward), GCC ATG TAG GCC ATG AGG TC (GAPDH-reverse), AAA CAG TCT ATT TCG GCG AAT C (TMEM126A-forward), GGG ATC ACA GCC ATT GGT AA (TMEM126A-reverse), GGG ATG AAT TGG TCA TAG CC (IL-1β-forward), ATG TGC TGG TGC TTC ATT CA (IL-1β-reverse), CAA GGA CAC AGA CTC AGC CA (TN-C-forward), GTT AAC GCC CTG ACT GTG GT (TN-C-reverse), CCG GAG AGG AGA CTT CAC AG (IL-6-forward), CAG AAT TGC CAT TGC ACA AC (IL-6-reverse), CTG CCT CAG CCT TTG ATT GT (M-CSF-forward), CCT TCC TCT CTC CCT TCC AC (M-CSF-reverse). Each sample was run in triplicate and threshold cycle (C_t) values were averaged for each reaction. Expression of each gene of interest was quantified as ΔC_t and normalized with the ΔC_t of the housekeeping gene, GAPDH. Amplification specificity was confirmed in each run by analyzing the melting temperature of the PCR product. Negative controls were run without cDNA templates. cDNA synthesis and quantitative PCR were performed in triplicate.

2.14. Statistical analyses

All statistical analyses were performed using Prism 5.0 software (GraphicPad, San Diego, CA). Parametric statistical analysis (mean and SEM) was performed by standard methods. For the analysis of differences between groups we used a non-parametric unpaired t-test. $P<0.05$ was considered significant.

3. Results

3.1. TMEM126A associates and co-localizes with CD137L in HEK 293 cells

To identify proteins that bind to CD137L, we performed yeast two-hybrid experiments and identified several potential binding partners. Four independent positives from the X-alpha gal assay turned out to contain the same mouse gene, TMEM126A (data not shown). To confirm that TMEM126A binds to CD137L, we over-expressed Flag-tagged CD137L and Myc-tagged TMEM126A in HEK 293 cells (Fig. 1A) and immunoprecipitated Flag-CD137L with anti-Flag Ab or Myc-TMEM126A with anti-Myc Ab. The immunoprecipitate was fractionated by SDS-PAGE, transferred to nitrocellulose membranes and blotted with anti-Myc Ab or anti-Flag Ab, respectively. As shown in Fig. 1B, Myc-TMEM126A was co-immunoprecipitated with Flag CD137L. Co-localization of CD137L with TMEM126A was further demonstrated by immunofluorescence microscopy (Fig. 1C). To map the binding domain of CD137L with TMEM126A, we over-expressed Flag-tagged full length (1–309 aa), N-terminal (1–81 aa), C-terminal (108–309 aa) CD137L and Myc-tagged TMEM126A, and immunoprecipitated Myc-TMEM126A with anti-Myc Ab and then blotted with anti-Flag Ab. As shown in Fig. 1D, the CD137L-C terminal was much better to be co-immunoprecipitated with Myc-TMEM126A than CD137L-full length. Sufficient amount of Flag-N-terminal CD137L protein could not obtained probably due to its structural instability in our expression conditions (Fig. 2D). These data indicate that TMEM126A binds to CD137L in HEK 293 cells over-expressing recombinant TMEM126A and CD137L, and suggest that the extracellular domain of CD137L may be, at least in part, involved in binding to TMEM126A because CD137L is a type 2 transmembrane protein.

3.2. Endogenous TMEM126A co-localizes with endogenous CD137L in RAW264.7 myeloid cells

To confirm that TMEM126A associates with CD137L in endogenous conditions, we used RAW264.7 cells, a mouse myeloid cell line that expresses CD137L constitutively and produces reverse signals by cross-linking of CD137L with rCD137-Fc [11]. CD137L was recovered more efficiently by pull-down than by immunoprecipitation (Fig. 2A), and TMEM126A was detected by Western blotting (Fig 2B, left) in the RAW264.7 cells. TMEM126A was previously reported to be of low molecular weight in liver cells and we similarly visualized a ~22 kDa protein in a mouse liver lysate. However, anti-TMEM126A pAb (Y-12) detected a ~66 kDa protein in RAW 264.7 cells and T cell hybridomas. To confirm the 66 kDa protein is related with TMEM126A, we made a rabbit anti-TMEM126A pAb using the N-terminal peptide of TMEM126A. Several bands were detected by Western blot analysis using this TMEM126A-N pAb. A band of 66 kDa protein was disappeared as Western blot analysis was done using the TMEM126A-N pAb pre-incubated with the N-terminal peptide of TMEM126A (Fig. 2B, right).

To examine whether TMEM126A binds to CD137L in the RAW264.7 cells, the CD137L was pulled-downed with rCD137-Fc and Western blot analysis was done using anti-TMEM126A pAb (Y-12). A band of 66 kDa protein was detected, and the intensity of the band was reduced by pre-incubation with the Ab-blocking peptide that had been used for making the anti-TMEM126 pAb (Y-12) (Fig. 2C, left). Immunoprecipitated CD137L with anti-CD137L also contained the 66 kDa TMEM126A protein (Fig. 2C, right). This indicates that a 66 kDa TMEM126A protein associates with CD137L in the RAW264.7 cells. We also examined the location of endogenous CD137L and TMEM126A by immunofluorescence microscopy. As shown in Fig. 2D, a fraction of TMEM126A co-localized with CD137L in unstimulated cells. In cells treated with hIgG-Fc, (as a control) TMEM126A was located inside the cell and CD137L was found both inside and outside the cell. On the other hand, in cells stimulated by cross-linking CD137L with rCD137-Fc TMEM126A and CD137L co-localized predominantly at the membrane of the cells. These data confirm that TMEM126A binds to and co-localizes with CD137L in RAW264.7 cells.

3.3. Transfection of myeloid cells with TMEM126A siRNA reduces CD137L–mediated functional responses

To examine the role of TMEM126A in CD137L reverse signaling, we transiently transfected RAW264.7 cells or thioglycollate-elicited peritoneal macrophages with siRNA against TMEM126A, and tested the effect on the CD137L signal-evoked cellular responses previously reported [11, 12]. Transfection of RAW264.7 cells with TMEM126A siRNA blocked tyrosine phosphorylation enhanced by CD137L ligation by rCD137-Fc (Fig. 3A), and inhibited CD137L-induced up-regulation of TN-C, IL-1β and M-CSF (Fig. 3B). In thioglycollate-elicited peritoneal macrophages, transfection with TMEM126A siRNA inhibited the CD137L-mediated down-regulation of IL-1β and IL-6 expression (Fig. 4).

3.4. CD137L reverse signaling is abolished in TMEM126A-deficient RAW264.7 myeloid cells

To confirm the role of TMEM126A in CD137L reverse signaling, we established a stable TMEM126A-deficient RAW 264.7 cell line, and tested the effect of cross-linking CD137L with either rCD137-Fc or anti-CD137L mAb (TKS-1) in these cells. Reduced expression of TMEM126A in TMEM126A-deficient RAW264.7 cells was confirmed by Western blotting as well as flow cytometry (Fig 5A). Detection of TMEM126A on cell surface by flow cytometery supports the idea that TMEM126A may associate with the extracellular domain of CD137L as shown in Fig. 1D. As shown in Fig. 5A, the knockdown of TMEM126A diminished tyrosine phosphorylation induced by CD137L ligation with rCD137-Fc (top left) or TKS-1 (top right). Cell adherence evoked by CD137L ligation with rCD137-Fc (Fig. 5B,

and 5D left) or TKS-1 (Fig. 5C, and 5D right) was almost completely blocked in TMEM126A-deficient RAW264.7 cells. These data indicate that TMEM126A plays a critical role in CD137L-mediated reverse signaling in myeloid cells.

4. Discussion

In the present work we presented novel findings related to proximal CD137L reverse signaling in mouse myeloid cells. We showed that TMEM126A associated and co-localized with CD137L in both 293 HEK cells over-expressed with recombinant proteins and murine myeloid RAW264.7 cells. We also showed that knockdown of TMEM126A abrogated both CD137L reverse signal transduction and its cellular functions. These data indicate that CD137L-mediated reverse signaling involves the association of TMEM126A with CD137L although it needs further studies to elucidate molecular mechanisms how TMEM126A relays CD137L reverse signaling. We do not yet know the precise cellular location of TMEM126A required for transmitting CD137L reverse signaling. Based on the data showing that TMEM126A binds to the extracellular domain of CD137L and is detected on cell surface by flow cytometry, cell surface expression of TMEM126A may be essential for CD137L reverse signaling. This notion is supported by the data that TMEM126A and CD137L co-localized predominantly on cell surface upon cross-linking CD137L. Kang et al. reported that CD137L associates with TLR4 and that CD137L is required to sustain LPS-induced TNF release in murine macrophages [15]. It is possible that TMEM126A may play a role in the association of CD137L with TLR4. Recently, we have found that LPS-induced TMEM126A expression on cell surface continues to increase with time whereas LPS-induced CD137L expression concomitantly decreases (manuscript in preparation). It implies that TLR4 signaling regulates cell surface expression of CD137L and TMEM126A.

The murine *TMEM126A* gene is located on chromosome 7, and its transcript (794 bp) contains one non-coding and four coding exons. TMEM126A protein consists of 196 amino acids and is predicted to have four transmembrane domains with the N-terminal and C-terminal sequences on the outside of the membrane. It belongs to the DUF1370 family of proteins, which consists of several hypothetical eukaryotic proteins of around 200 residues. Members of this family seem to be specific to mammals and their function is unknown. Two recent studies have reported that TMEM126A is mutated in inherited optic and auditory neuropathy [16, 17], but our data represent the first report of TMEM126A expression and activity in lymphoid cells.

TMEM126A was reported to be of low molecular weight and using an anti-TMEM126A pAb (Y-12), we detected a protein of about 22 kDa in mouse liver cells. However, in RAW264.7 cells, a T cell hybridoma, and splenocytes, the same Ab detected a 66 kDa protein. Pre-incubation of the anti-TMEM126A pAb (Y-12) with the blocking peptide used to generate the antibody reduced the intensity of the 66 kDa band (Fig. 2C). Importantly we found that when we transfected cells with TMEM126A siRNA or shRNA, the amount of the 66 kDa protein was decreased as much as TMEM126A transcripts (Fig. 3A and Fig. 5A), and we detected a 66 kDa protein in RAW264.7 cells with another anti-TMEM126A-N pAb, (Fig. 2B, right). Nucleotide sequences bound with antisense siRNA and shRNA against TMEM126A and the sequence of amino acids in a peptide for making the anti-TMEM126A-N pAb are shown (Supplement Fig. 1). These observations indicate that the 66 kDa protein is indeed TMEM126A although we do not know how the 66 kDa form of TMEM126A is regulated. We speculate that TMEM126A may trimerize to relay CD137L reverse signals since CD137L is a trimer [18].

5. Conclusions

In summary, we have demonstrated that in mouse myeloid cells CD137L reverse signal-mediated cellular responses involve TMEM126A. This is the first study demonstrating a role of TMEM126A in the immune system.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

TMEM126A	transmembrane protein 126A
TNF(R)	Tumor Necrosis Factor (Receptor)
M-CSF	macrophage-colony stimulating factor
APC	antigen presenting cell
TN-C	tenascin C
PBS	phosphate buffered saline
mAb	monoclonal antibody
pAb	polyclonal antibody
RT-PCR	reverse transcription-polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate– polyacrylamide gel electrophoresis

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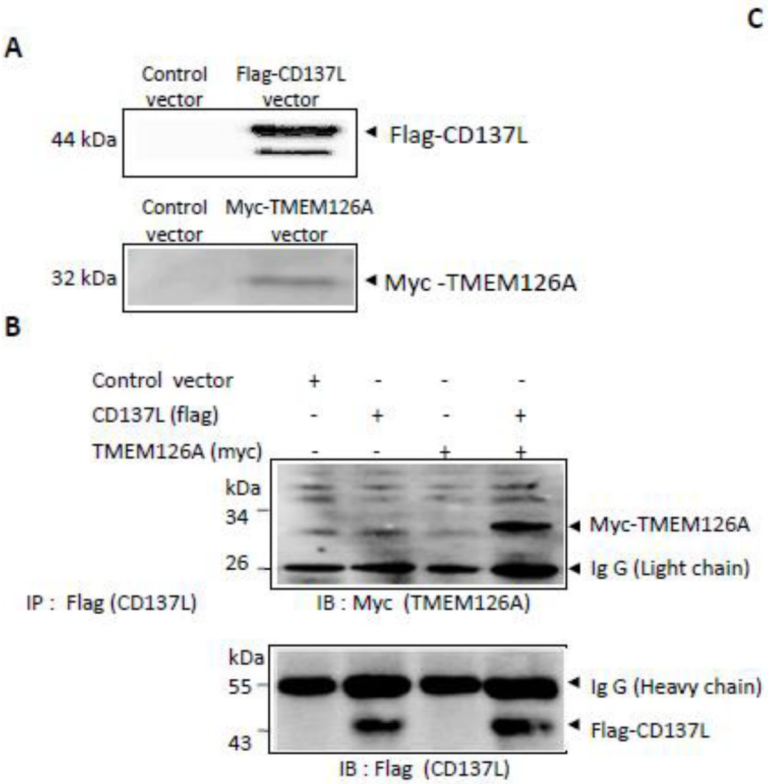
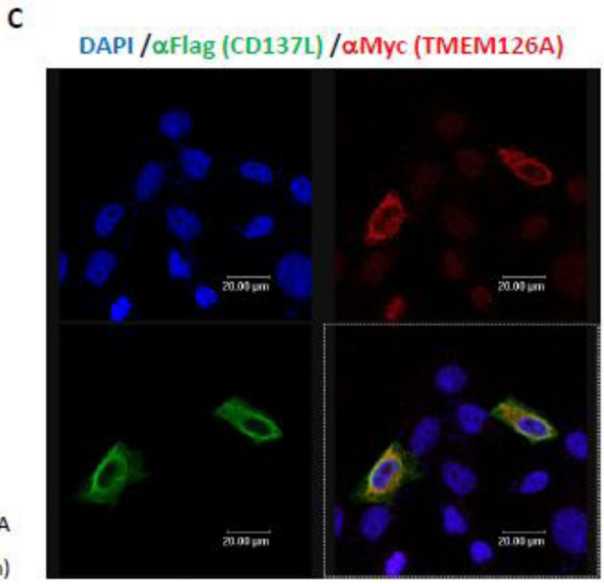
Highlights

- The aim of this study was to elucidate the reverse signaling pathways of CD137 ligand in myeloid cells.
- We have novel findings that in mouse myeloid cells CD137L reverse signal-mediated cellular responses involve TMEM126A.
- This is the first study demonstrating a role of TMEM126A in the immune system.





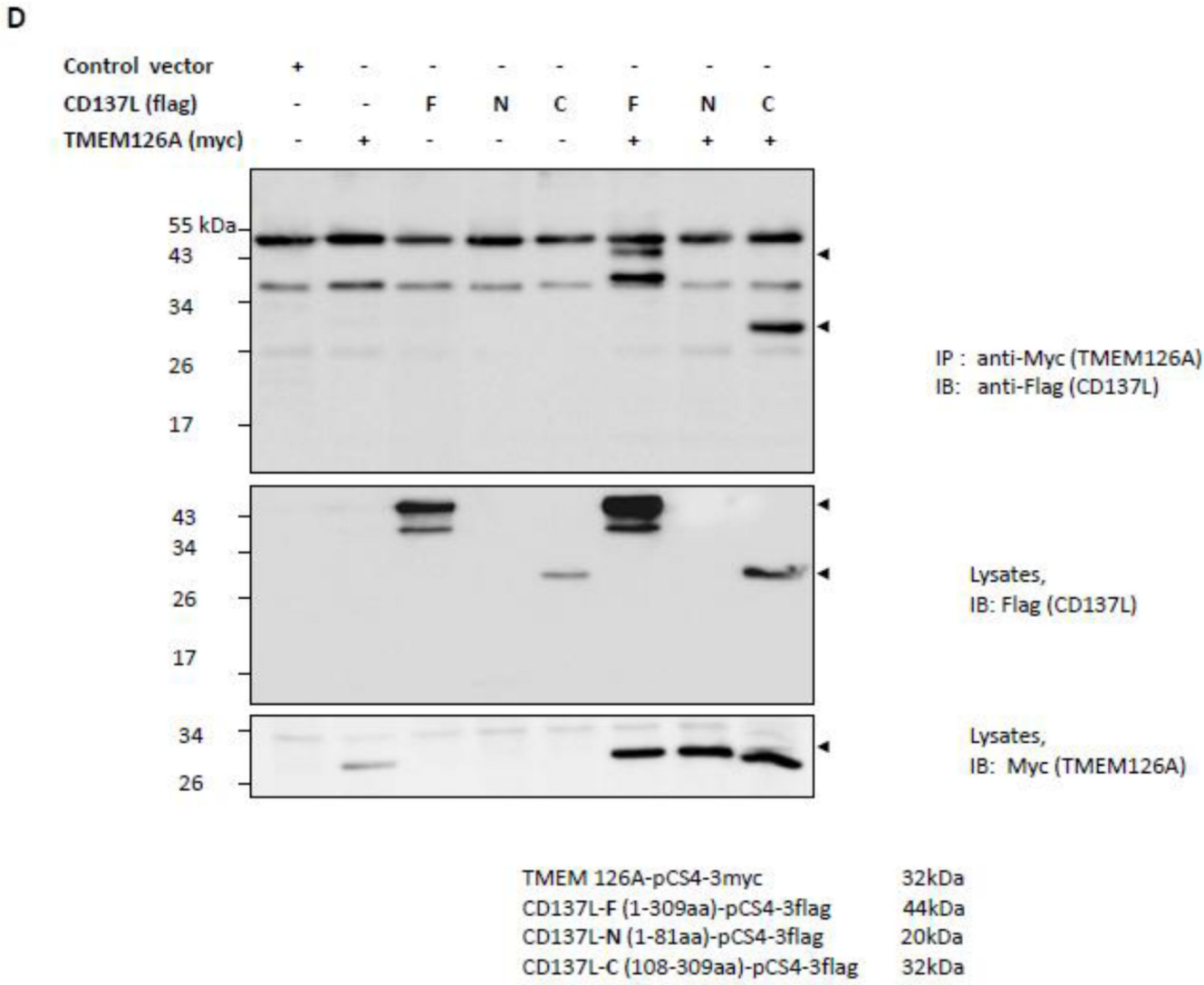
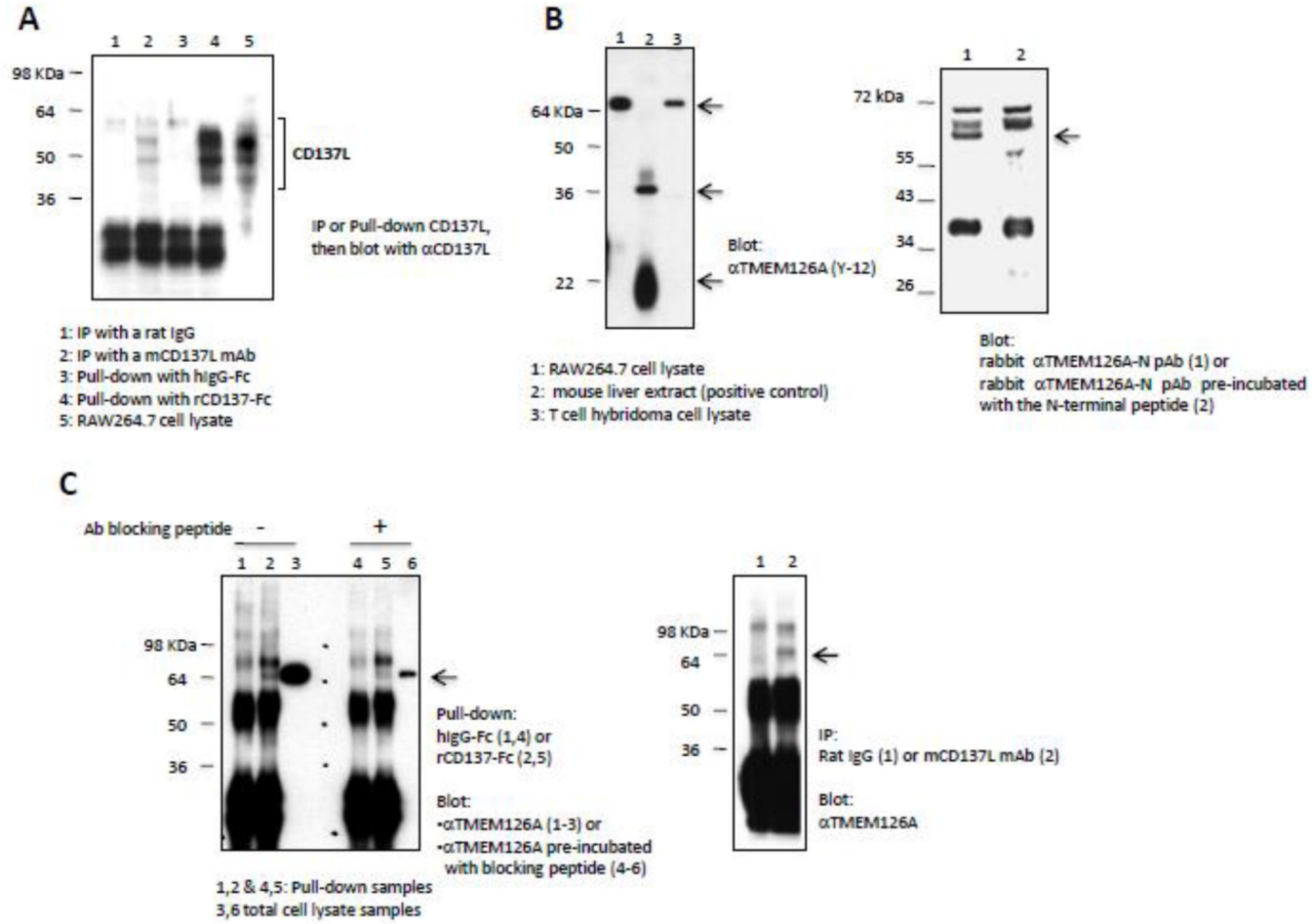


Fig. 1.

TMEM126A binds to CD137L in HEK 293 cells over-expressing tagged TMEM126A and CD137L. (A) HEK 293 cells were transfected with control vector or flag-CD137L and myc-TMEM126A vectors. Equal amounts of protein were fractionated by SDS-PAGE and transferred to nitrocellulose membranes, and CD137L was detected with anti-Flag Ab. After stripping the membranes, TMEM126A was detected by re-probing with anti-Myc Ab. Similar results were obtained in three independent experiments. (B) HEK 293 cells were transfected with control vector, and flag-CD137L and/or myc-TMEM126A vectors. Cell lysates were immunoprecipitated with anti-Flag Ab, and the immunoprecipitated proteins were washed, separated by SDS-PAGE and transferred to nitrocellulose membranes. TMEM126A was detected with anti-Myc Ab (top). The blots were stripped and reprobed with anti-Flag Ab (bottom) to detect CD137L. Similar results were obtained in three independent experiments. (C) HEK 293 cells were transfected with both flag-CD137L and myc-TMEM126A, stained with DAPI, anti-Flag Ab and anti-Myc Ab and dual-visualized by confocal microscopy. Similar results were obtained in four independent experiments. (D) HEK 293 cells were transfected with control vector, flag-CD137L-F, flag-CD137L-N, flag-CD137L-C and/or myc-TMEM126A vectors. Cell lysates were immunoprecipitated with

anti-Myc Ab (top), and the immunoprecipitated proteins were washed, separated by SDS-PAGE and transferred to nitrocellulose membranes. TMEM126A was detected with anti-Flag Ab (top). CD137L (middle) and TMEM126A (bottom) in cell lysates were detected with anti-Flag Ab or anti-Myc Ab, respectively. Similar results were obtained in three independent experiments.



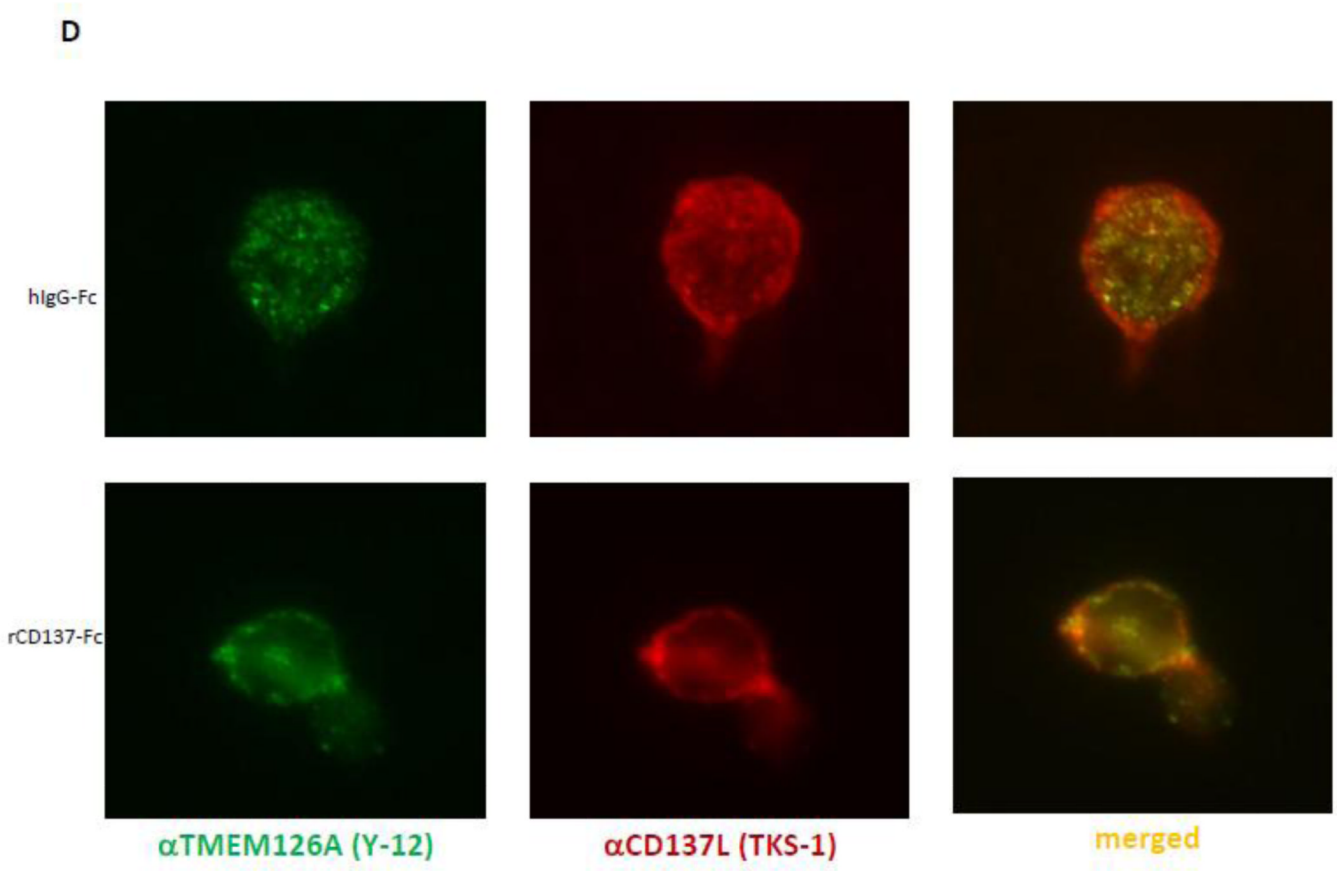
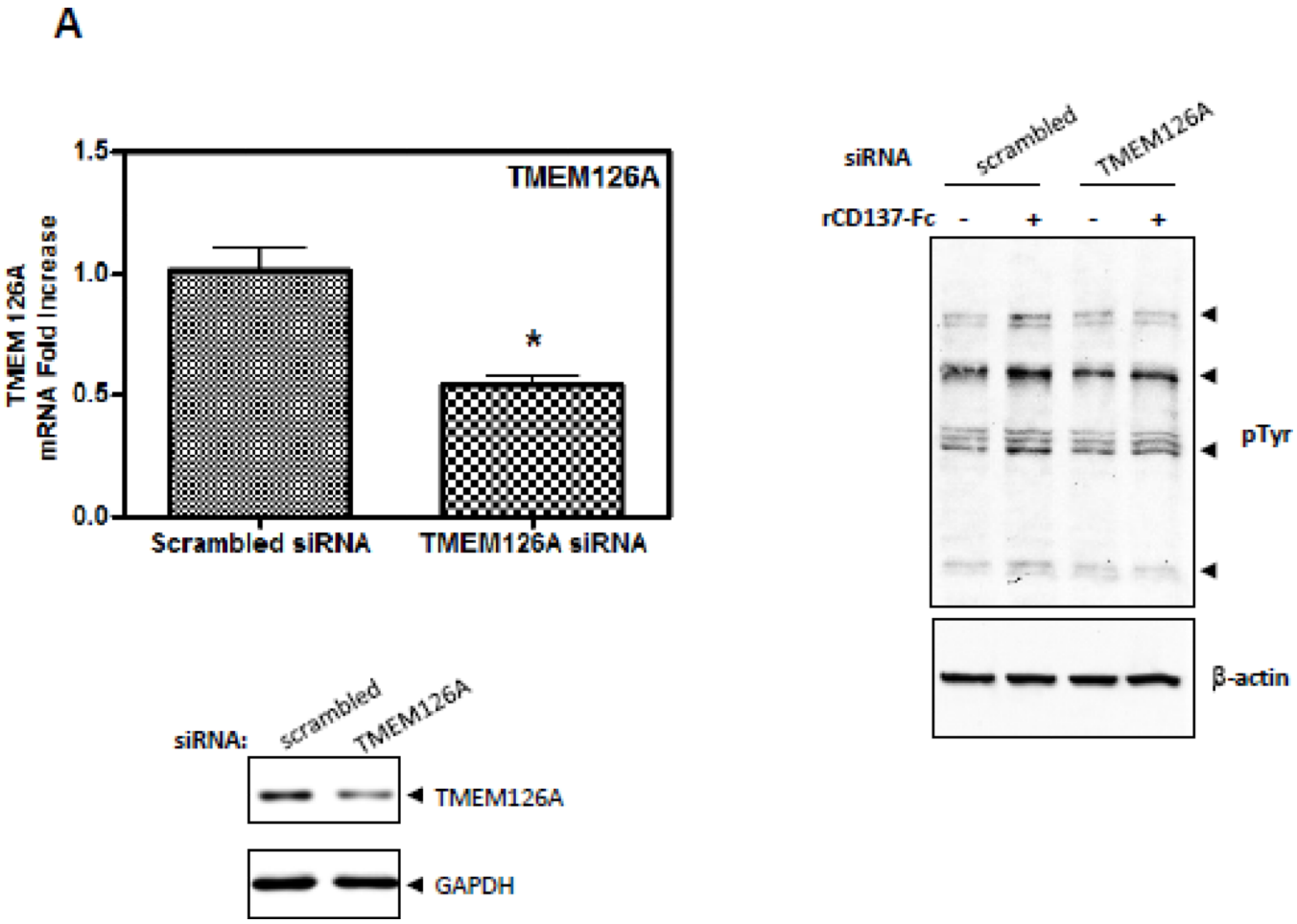


Fig. 2.

Endogenous TMEM126A and CD137L co-localize in murine myeloid RAW264.7 cells. (A) Cell lysates of RAW264.7 cells were immunoprecipitated with rat IgG or anti-CD137L mAb (lanes 1 and 2), or pulled-down with hIgG-Fc or rCD137-Fc (lanes 3 and 4). Immunoprecipitated proteins were washed, separated by SDS-PAGE and transferred to nitrocellulose membranes. CD137L was detected with the anti-CD137L pAb. Similar results were obtained in three independent experiments. (B) Cell or tissue lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. (Left panel) TMEM126A was detected with the anti-TMEM126A pAb (Y-12). (Right panel) TMEM126A was detected with anti-TMEM126A-N pAb in the absence (lane 1) or presence (lane 2) of Ab blocking peptide. Similar results were obtained in three independent experiments. (C, left panel) Lysates of RAW264.7 cells were pulled-down with hIgG-Fc (lane 1 and 4) or rCD137-Fc (lane 2 and 5). The pulled-down proteins were washed, separated by SDS-PAGE and transferred to nitrocellulose membranes. TMEM126A was detected with anti-TMEM126A pAb (Y-12) in the absence (lane 1~3) or presence (lane 4~6) of Ab blocking peptide. (C, right panel) Lysates of RAW264.7 cells were immunoprecipitated with isotype control rat IgG (lane 1) or anti-CD137L mAb (lane 2). The immunoprecipitated proteins were washed, separated by SDS-PAGE and transferred to nitrocellulose membranes. TMEM126A was detected with anti-TMEM126A pAb (Y-12). Similar results were obtained in three independent experiments. (D) RAW263.7 cells were incubated on cover glasses coated with hIgG or rCD137-Fc for 30 min. Immunofluorescence staining was performed as described in “Materials and Methods”. Cells were stained with both anti-CD137L Ab (TKS-1) and anti-TMEM126A Ab (Y-12) and dual-visualized by immunofluorescence microscopy. Similar results were obtained in four independent experiments.



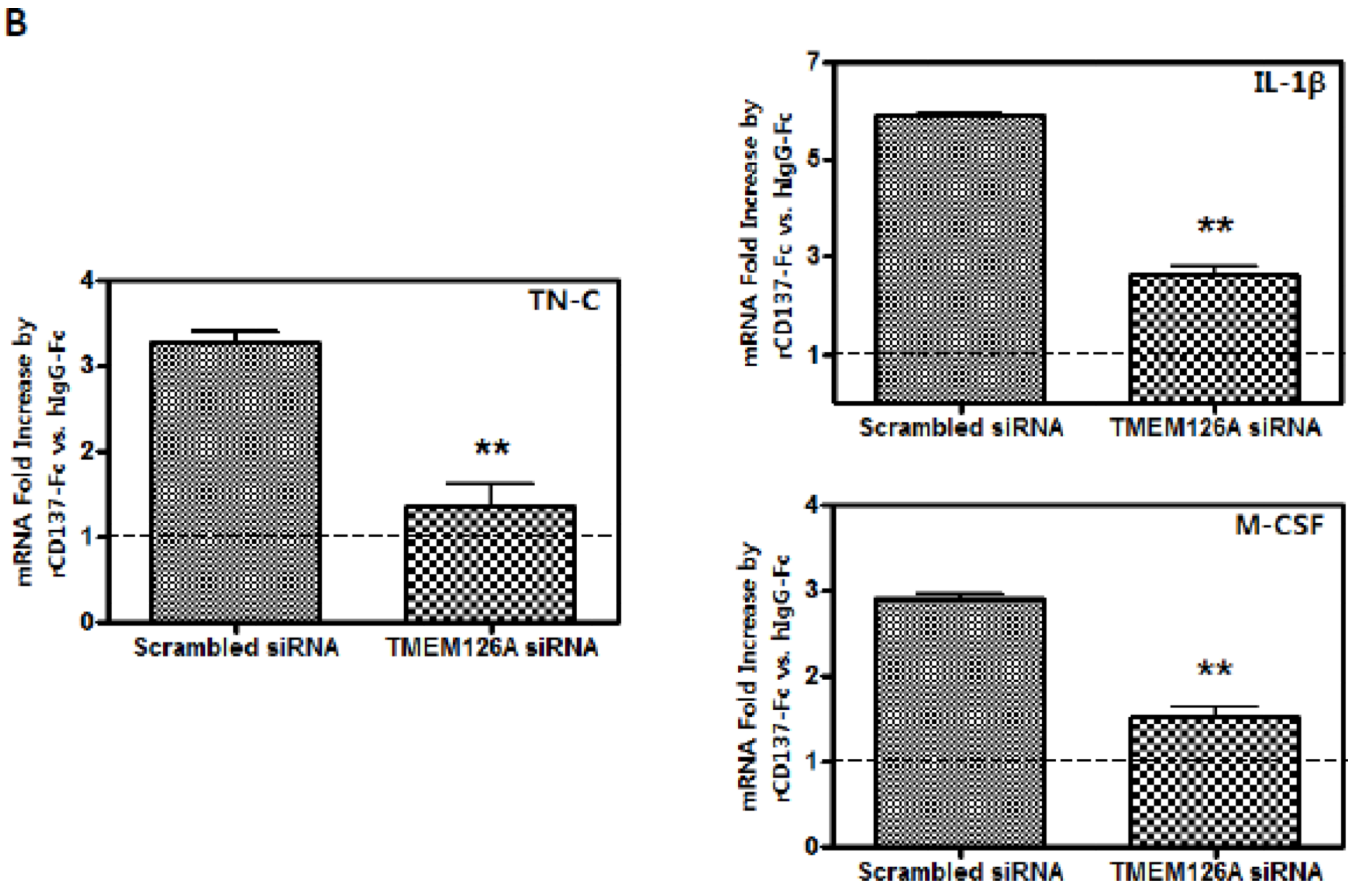


Fig. 3.

Transfection of RAW264.7 cells with TMEM126A siRNA abrogates CD137L reverse signal-induced up-regulation of TN-C, IL-1β and M-CSF. (A, left panel) RAW264.7 cells were transfected with 20 nM scrambled siRNA or TMEM126A siRNA in the presence of 100 ng/ml hIgG or rCD137-Fc for 2 days. Total RNA was isolated and cDNA was synthesized. Quantitative real time-PCR was performed using primer pair specific for TMEM126A gene, as described in "Materials and Methods" (left top). Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. TMEM126A was detected with the anti-TMEM126A pAb. Similar results were obtained in three independent experiments (left bottom). (A, right panel) RAW264.7 cells were transfected with 20 nM scrambled siRNA or TMEM126A siRNA for 2 days. Cells were re-plated and cultured on hIgG- or rCD137-Fc-coated dishes for additional one hour. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. pTyr proteins was detected with the anti-pTyr mAb (4G10 platinum). Similar results were obtained in three independent experiments. (B) RAW264.7 cells were transfected with 20 nM scrambled siRNA or TMEM126A siRNA in the presence of 100 ng/ml hIgG or rCD137-Fc for 2 days. Total RNA was isolated and cDNA was synthesized. Quantitative real time-PCR was performed using primer pairs specific for TN-C, IL-1β and M-CSF genes, as described in "Materials and Methods". Results are representative of three independent experiments * $p < .05$, ** $p < .001$, compared with the corresponding control group, using Student's two-tailed t-test.

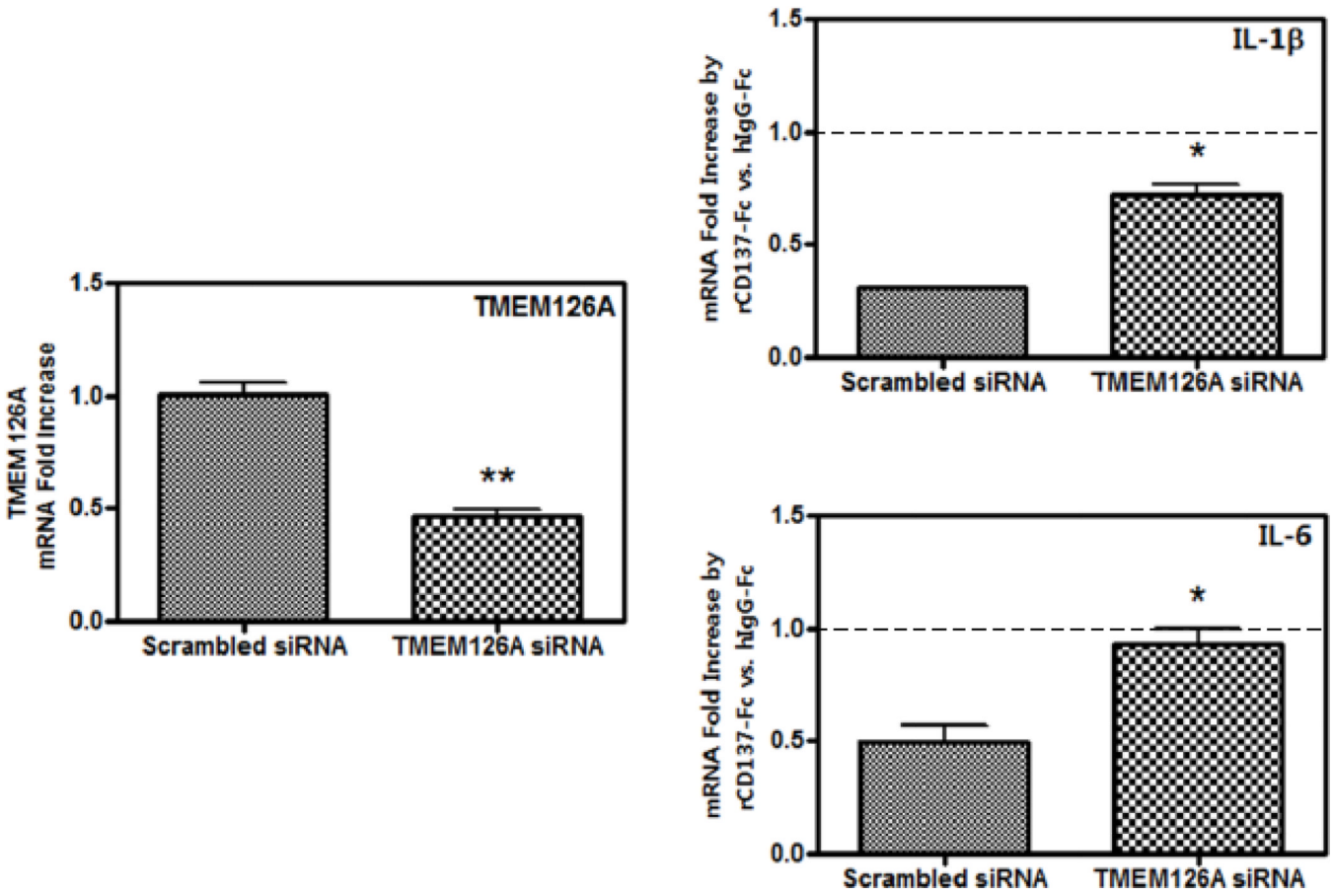
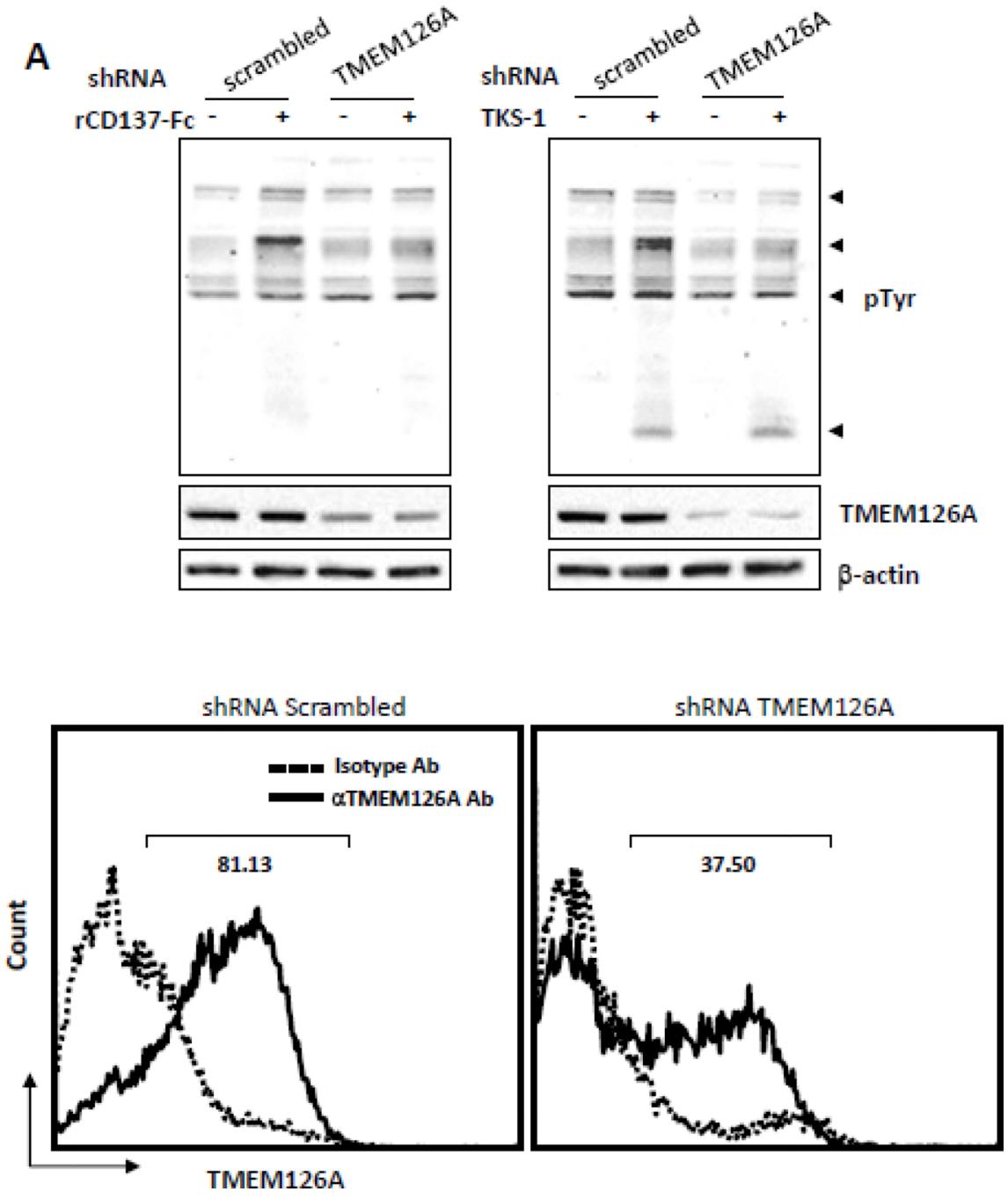
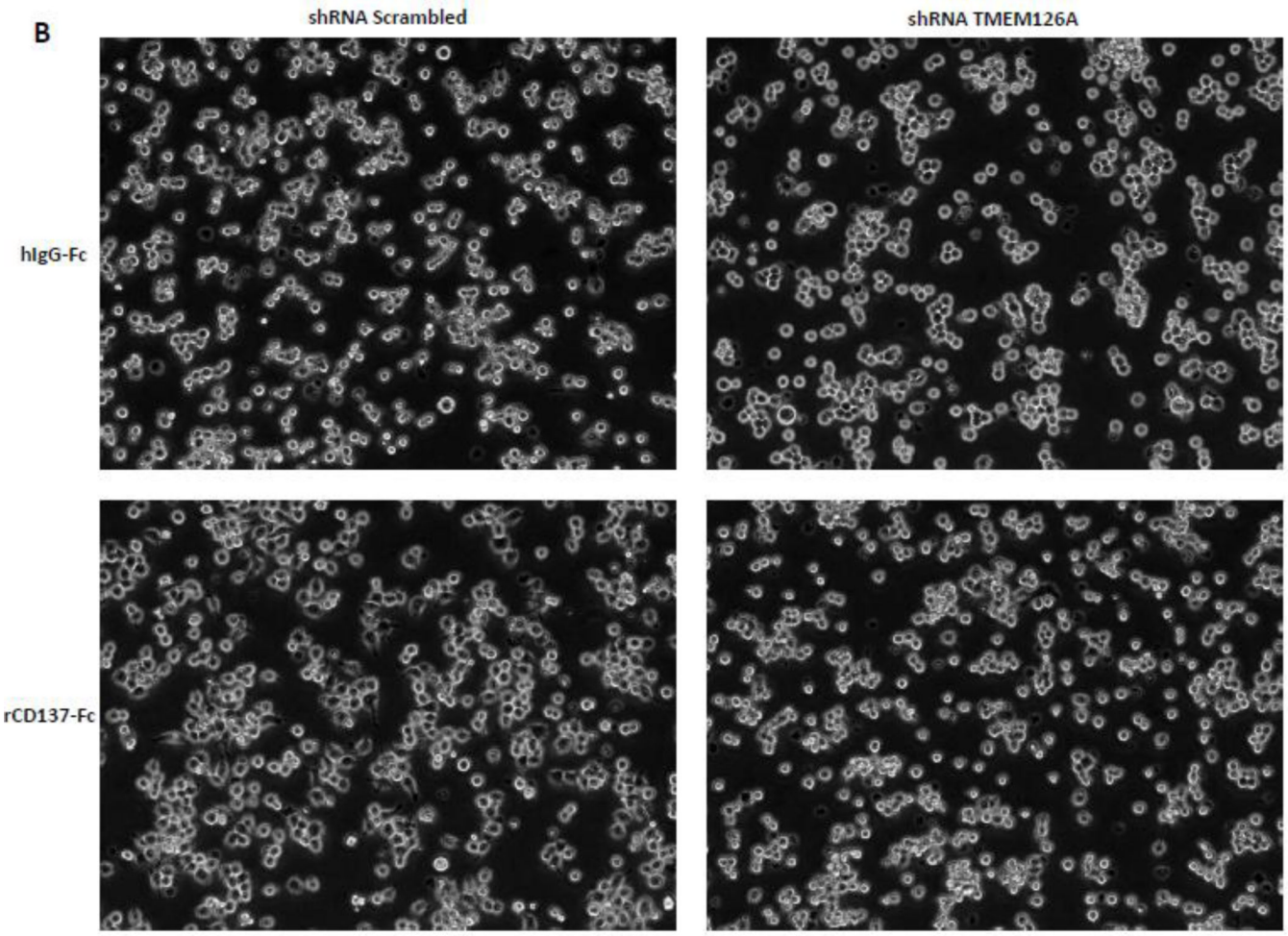


Fig. 4.

Transfection of thioglycollate-elicited peritoneal macrophages with TMEM126A siRNA inhibits CD137L reverse signal-induced down-regulation of IL-1β and IL-6. Thioglycollate-elicited peritoneal macrophages cells were transfected with 20 nM scrambled or TMEM126A siRNA in the presence of 100 ng/ml hIgG or rCD137-Fc for 2 days. Total RNA was isolated and cDNA was synthesized. Quantitative real time-PCR was performed using primer pairs specific for TMEM126A, IL-1β and IL-6 genes, as described in "Materials and Methods". Results are representative of three independent experiments * $p < .05$, ** $p < .001$, significantly different from corresponding control group, using Student's two-tailed t-test.





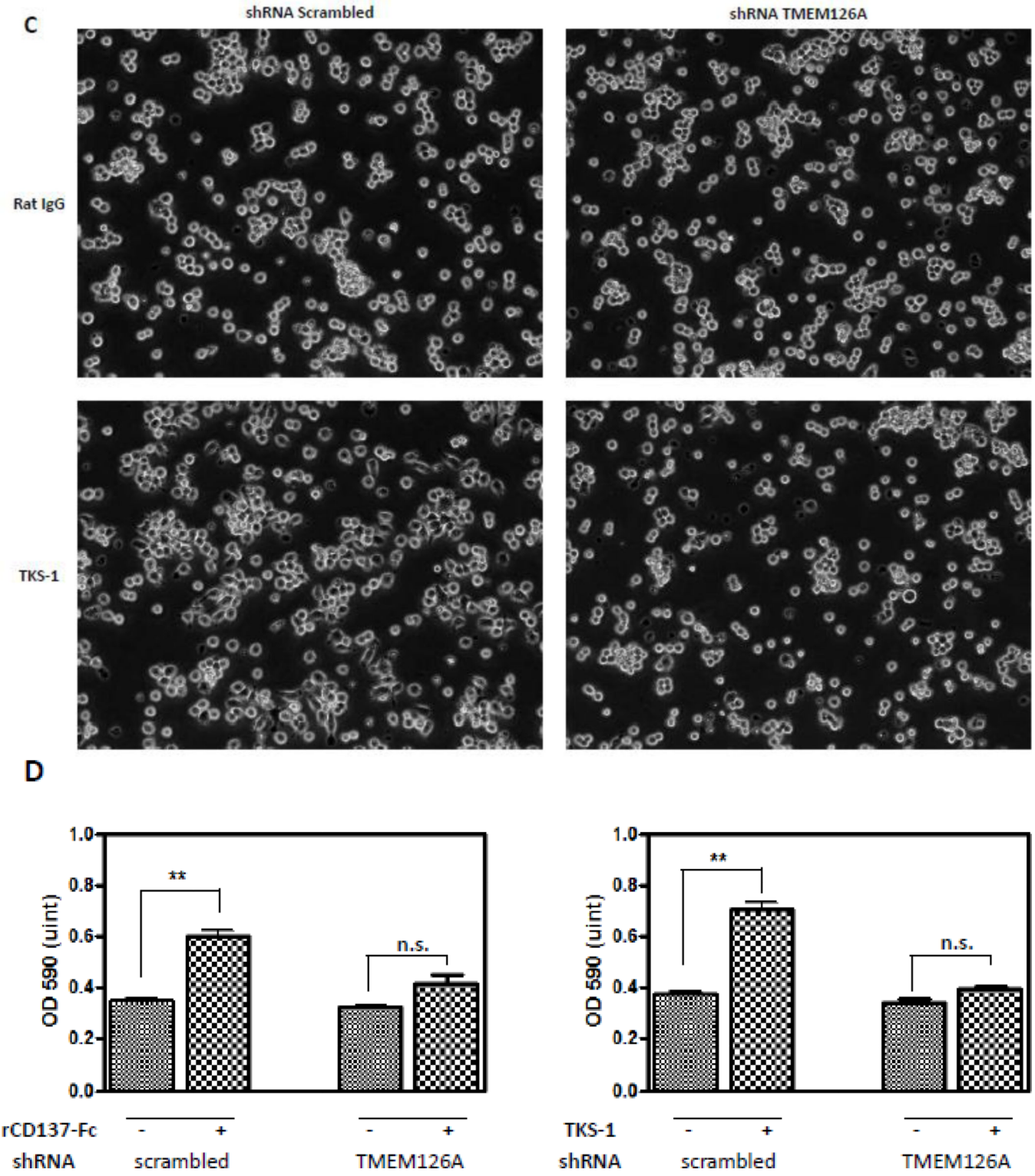


Fig. 5.

Stable transduction of RAW264.7 cells with retroviral TMEM126A shRNA reduces CD137L reverse signaling. (A) Retroviral scrambled or TMEM126A shRNA transduced-RAW264.7 cells were cultured on 100 ng/ml hIgG- or rCD137-Fc (top left) and 2.5 μg/ml

rat IgG-or TKS-1 (top right)-coated dishes for one hour. Cell lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes. pTyr proteins was detected with the anti-pTyr mAb (4G10 platinum). TMEM126A was detected by Western blotting (top) and flow cytometery (bottom). Similar results were obtained in three independent experiments. (B and C) Retroviral scrambled or TMEM126A shRNA transduced RAW264.7 cells were cultured on 100 ng/ml hIgG- or rCD137-Fc (B) and 2.5 μg/ml rat IgG- or TKS-1 (C)-coated dishes for one hour. Cells were visualized by microscopy. Results are representative of three independent experiments (D) Cell adherence was quantified as described in “Materials and Methods”. **$p < .001$, n.s., not significant, compared with the corresponding control group, using Student’s two-tailed t-test.