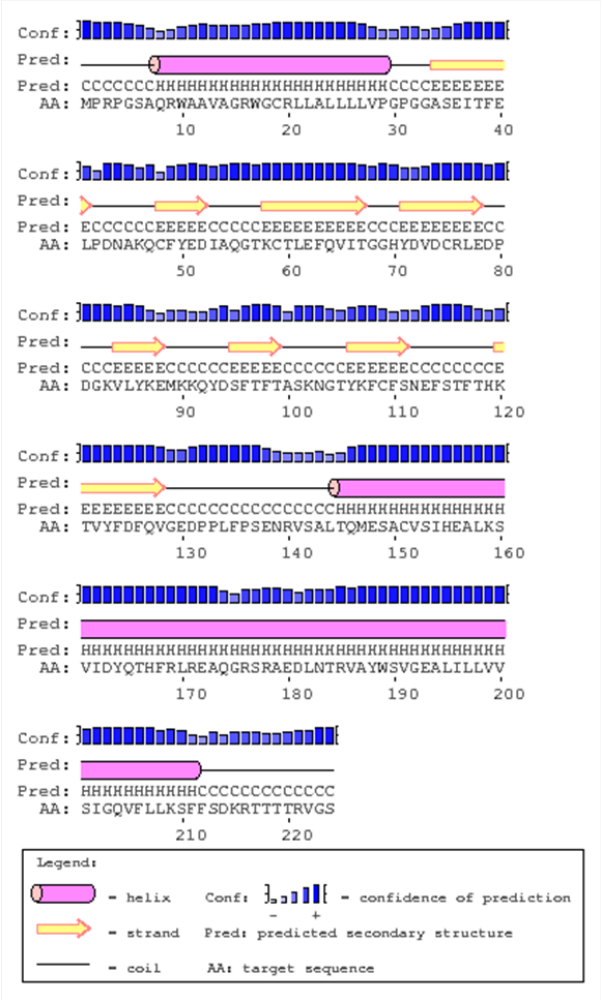
**A**

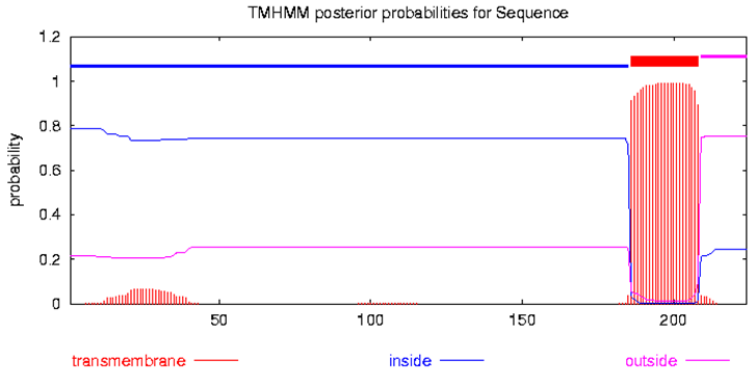



**B**

## TMHMM result

HELP with output formats

```
# Sequence Length: 224
# Sequence Number of predicted TMHs:  1
# Sequence Exp number of AAs in TMHs: 23.85121
# Sequence Exp number, first 60 AAs:  1.32782
# Sequence Total prob of N-in:        0.78565
Sequence        TMHMM2.0        inside       1   185
Sequence        TMHMM2.0        TMhelix    186   208
Sequence        TMHMM2.0        outside    209   224
```



**C**

```
SP|sp|Q9Y3B3|TMED7_HUMAN|TMED7_HUMAN   MPRPGSAQRWAAVAGRWGCRLLAL--LLLVPGPGGASEITFELPDNAKQCFYEDIAQGTK 58
SP|sp|D3ZTX0|TMED7_RAT|TMED7_RAT       MPRPRSATCWAAAAGRWSCRLLALLLLLLLPGPSGGSEITFELPDNAKQCFYEDITQGTK 60
TR|tr|D3YZZ5|D3YZZ5_MOUSE|D3YZZ5_MOUSE MPRPGSAPRWAAAAGRWGCRLLAL--LLLLPAPSGGSEITFELPDNAKQCFYEDITQGTK 58
TR|tr|H2QRC6|H2QRC6_PANTR|H2QRC6_PANTR MPRPGSAQRWAAVAGRWGCRLLAL--LLLVPGPGGASEITFELPDNAKQCFYEDIAQGTK 58
TR|tr|A4IFT6|A4IFT6_BOVIN|A4IFT6_BOVIN MPRLGSAPRWAAAAGRWGCRLLVLL-LFLVPGPGGASEITFELPDNAKQCFYEDITQGTK 59
TR|tr|Q6NWX3|Q6NWX3_DANRE|Q6NWX3_DANRE -MA-------GSSLSSWLLPLFV-QVVMMKVGLSSASELTFELPDNAKQCFYEDITIGTK 51
TR|tr|F1RLE5|F1RLE5_PIG|F1RLE5_PIG     MPRLGSAQRWAAAAGRWGHRLLVLL-LLLVPGPGGASEITFELPDNAKQCFYEDITQGTK 59
TR|tr|H9ESZ1|H9ESZ1_MACMU|H9ESZ1_MACMU MPRPGSAQRWAAAAGRWGCGLLAL--LLLVPGPGGASEITFELPDNAKQCFYEDIAQGTK 58
TR|tr|F6QDX4|F6QDX4_HORSE|F6QDX4_HORSE MLRPGSARRWLAAAGRWSCRLLALL--LLVPGPGGASEITFELPDNAKQCFYEDITQGTK 58
TR|tr|J9PB00|J9PB00_CANFA|J9PB00_CANFA MPGPGSAQRWAAAAGRWGCRLLALLLLLLLPGPGGASEITFELPDNAKQCFYEDITQGTK 60
                                        :  . *   *:.    ::  . ...**:***************: ***

SP|sp|Q9Y3B3|TMED7_HUMAN|TMED7_HUMAN   CTLEFQVITGGHYDVDCRLEDPDGKVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 118
SP|sp|D3ZTX0|TMED7_RAT|TMED7_RAT       CTLEFQVITGGHYDVDCRLEDPDGKVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 120
TR|tr|D3YZZ5|D3YZZ5_MOUSE|D3YZZ5_MOUSE CTLEFQVITGGHYDVDCRLEDPDGKVLYKEMKKQYDSFTFTASRNGTYKFCFSNEFSTFT 118
TR|tr|H2QRC6|H2QRC6_PANTR|H2QRC6_PANTR CTLEFQVITGGHYDVDCRLEDPDGKVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 118
TR|tr|A4IFT6|A4IFT6_BOVIN|A4IFT6_BOVIN CTLEFQVITGGHYDVDCRLEDPDGNVLYKEMKKQYDSYTFTASKNGTYKFCFSNEFSTFT 119
TR|tr|Q6NWX3|Q6NWX3_DANRE|Q6NWX3_DANRE CTLEFQVVTGGHYDVDCRLEDPEGTVLYKEMKKQYDSFTFSAARNGTYKFCFSNEFSTFT 111
TR|tr|F1RLE5|F1RLE5_PIG|F1RLE5_PIG     CTLEFQVITGGHYDVDCRLEDPDGNVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 119
TR|tr|H9ESZ1|H9ESZ1_MACMU|H9ESZ1_MACMU CTLEFQVITGGHYDVDCRLEDPDGKVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 118
TR|tr|F6QDX4|F6QDX4_HORSE|F6QDX4_HORSE CTLEFQVITGGHYDVDCRLEDPDGNVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 118
TR|tr|J9PB00|J9PB00_CANFA|J9PB00_CANFA CTLEFQVITGGHYDVDCRLEDPDGNVLYKEMKKQYDSFTFTASKNGTYKFCFSNEFSTFT 120
                                        *******:**************:*.************:**:*::***************
```

**Fig. S1. Bioinformatics analyses of TMED7 protein.** (**A**) Secondary structure prediction of TMED7 generated by PSIPRED v. 3.3. (**B**) Transmembrane domain prediction of TMED7 by TMHMM v. 2.0. Residues 186-208 were predicted to be part of the transmembrane helix with high confidence. The annotation 'inside' refers to extracellular or luminal whereas 'outside' is cytosolic. (**C**) Multiple amino acid sequence alignment of the TMED7 GOLD domain from different species generated using Clustal Omega. Residues in blue shade are the N-glycosylation motif Asn-Xaa-Ser/Thr where Xaa is any amino acid except P.

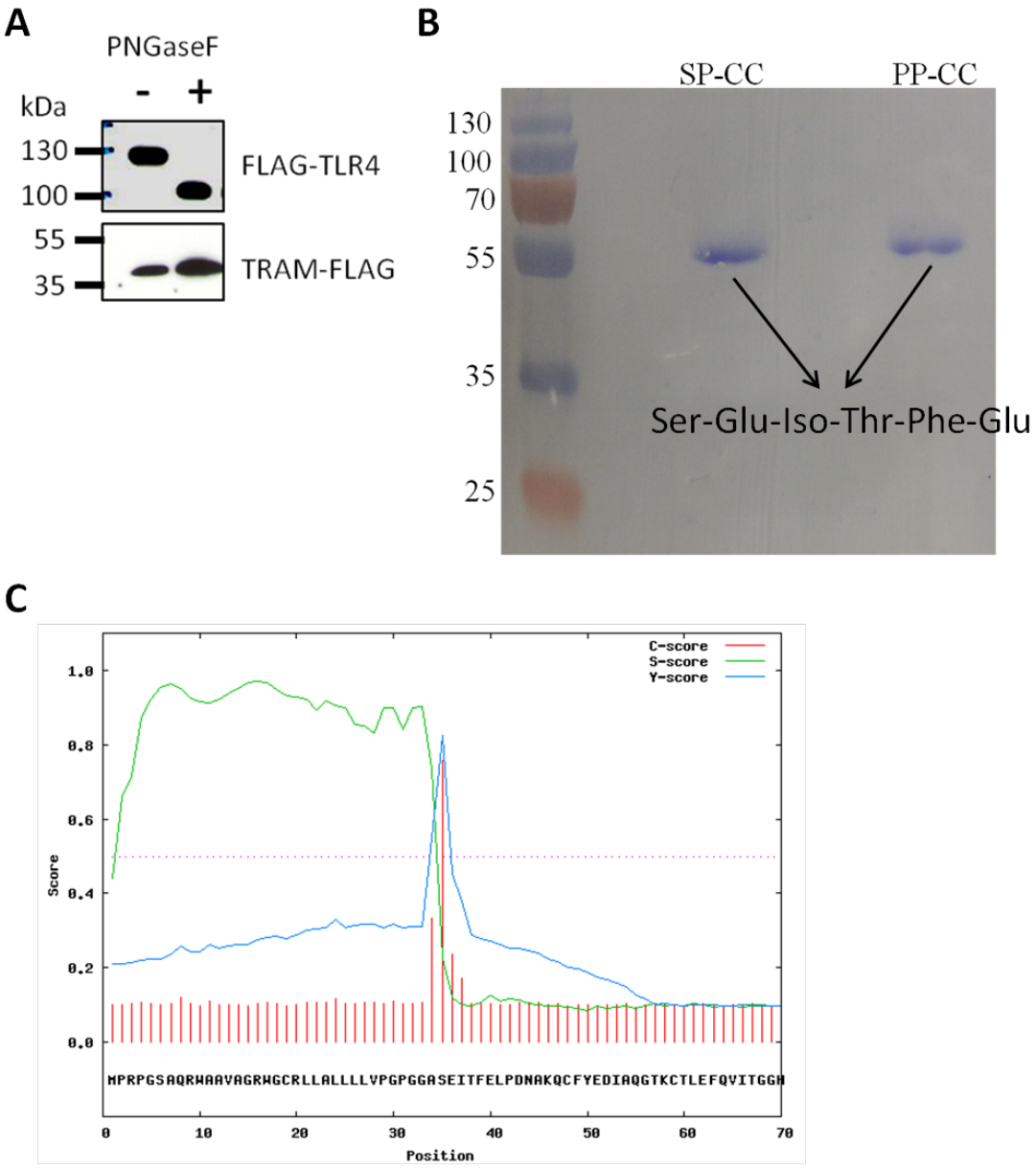



**Fig. S2. TMED7 is a type I transmembrane glycoprotein.** This figure is related to Figure 1. (**A**) FLAG-TLR4 and TRAM-FLAG were overexpressed in HEK293T cells and immunoprecipitated. Their glycosylation was confirmed by treatment with 1000 U PNGaseF and analyzed by Western blot. Western blots are representative of *n*=3. (**B**) Baculovirus expression of CC constructs with native (SP-CC) or PPTLS (PP-CC) signal peptide in *T.ni* insect cells. Protein was harvested and purified two days post infection, followed by transfer to PVDF membrane and sequenced by Edman degradation where the first six residues were identified (Ser-Glu-Iso-Thr-Phe-Glu). (**C**) Signal peptide prediction of TMED7 by SignalP V. 4.0 shows that the first 34 residues are a signal peptide with the mature protein starts at $Ser^{35}$.

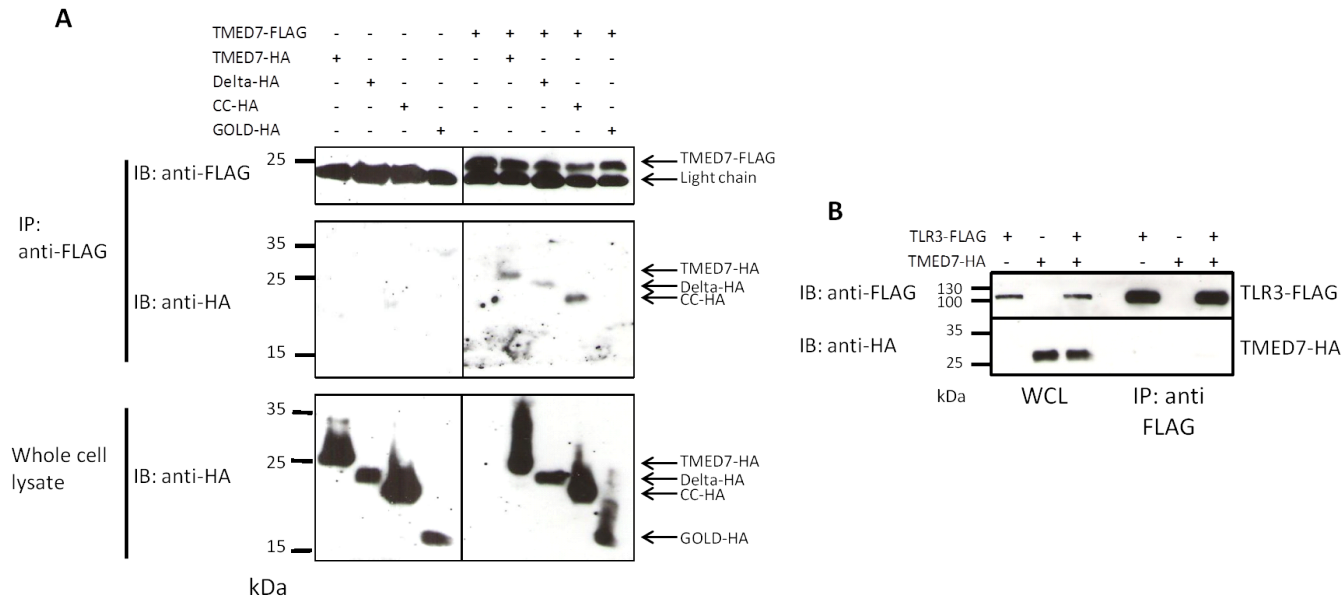



**Fig. S3. TMED7 oligomerization and interaction with TLR4.** This figure is related to Figure 2. (A) TMED7 requires the coiled-coil domain to oligomerize. THP-1 macrophages were transduced with lentivirus as shown in the figure to express HA-tagged TMED7 and the truncated mutants. Three days post transduction protein was harvested and immunoprecipitated. (B) TMED7 does not interact with TLR3. HEK293T cells were transiently transfected with plasmids as indicated in the figure and lysed two days post transfection. Overexpressed proteins were immunoprecipitated. In both experiments, immunoprecipitation was carried out using anti-FLAG M2 magnetic beads and analyzed using Western blot. Anti-HA antibody was used to detect the presence of co-immunoprecipitated proteins due to protein-protein interaction.WCL: whole cell lysate. Western blots are representative of $n$=3.

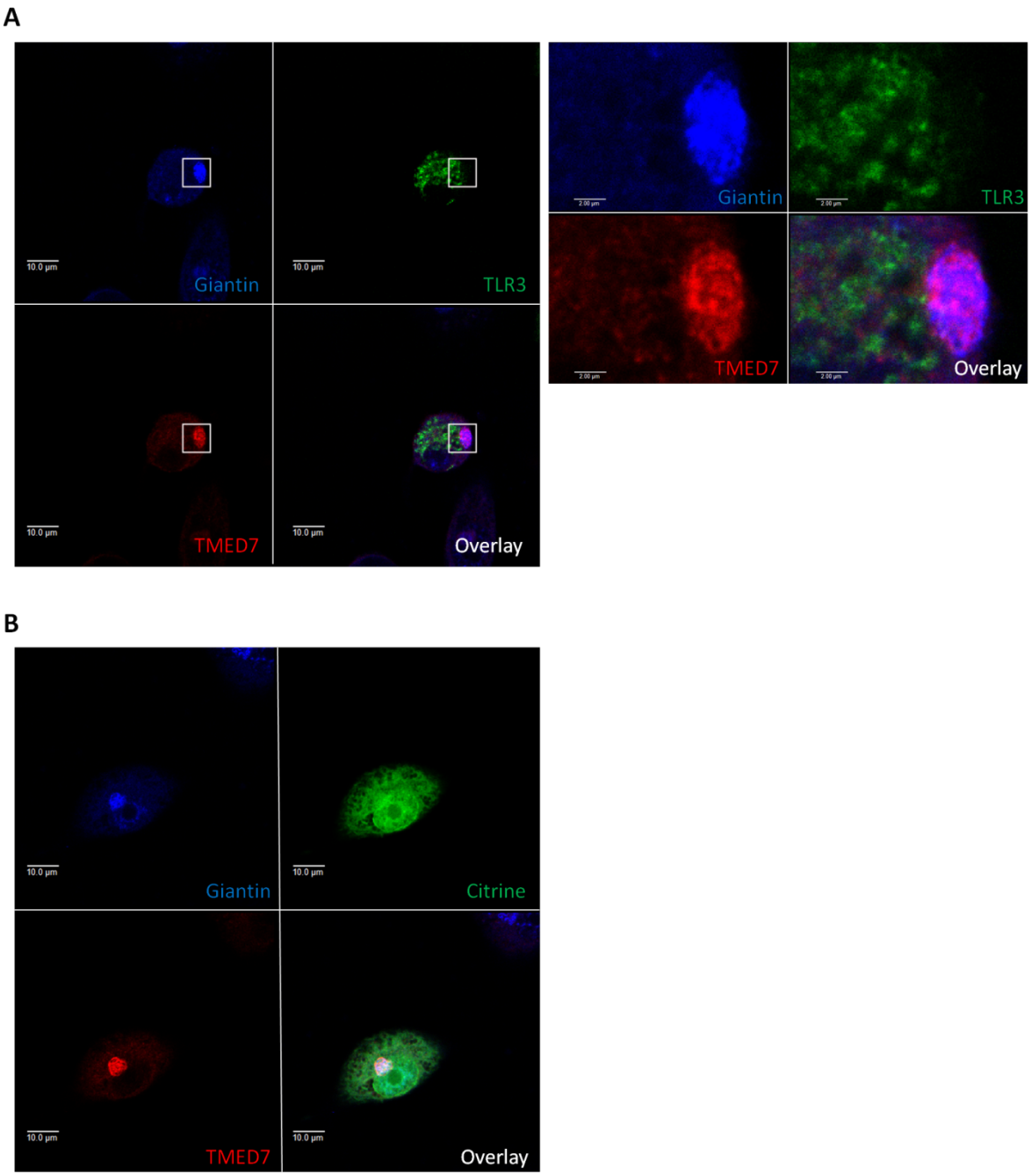



**Fig. S4. TMED7 does not colocalize with TLR3 in THP-1 macrophages.** THP-1 macrophages were transduced with lentivirus and incubated for three days. The cells were permeabilized and HA-tagged TMED7 were labelled with AlexaFluor633 (red) whereas transduced TLR3 was tagged with C-terminal mCitrine (green) (A). Right panel shows the zoomed area in the white box in left panel. (B) mCitrine on its own did not cololcalize with TMED7 in THP-1 macrophages. Approximately 20 cells were observed to have similar staining pattern in each condition from two independent experiments. Scale bar is 10 μm.

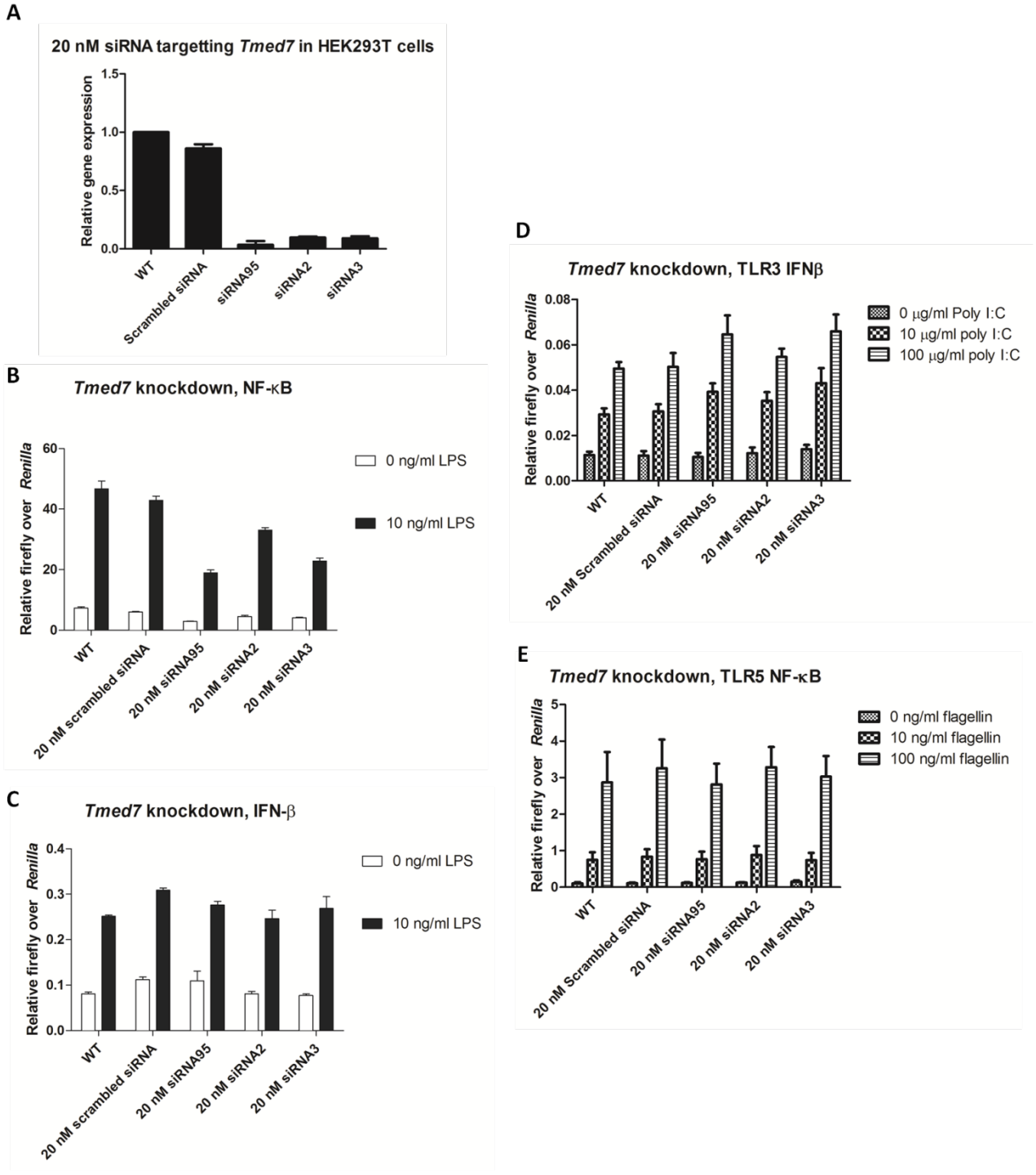



**Fig. S5. *Tmed7* knockdown specifically reduces MyD88-dependent TLR4 signaling.** This figure is related to Figure 4. In all panels, HEK293 cells were transfected with siRNA as indicated 3 days prior to DNA plasmid transfection. Cells were then transiently transfected with TLR4, MD2, CD14, phRG, and NF-κB luciferase (A) or TRAM-FLAG and IFNβ luciferase (B) and stimulated with LPS for 6 hours. (C) HEK293 cells were transfected with TLR3-FLAG, phRG, and IFNβ luciferase and stimulated with poly I:C for 6 hours. (D) HEK293 cells were transfected with phRG and NF-κB luciferase, and stimulated with flagellin for 6 hours. Data is presented as firefly luciferase activity normalized against *Renilla* luciferase activity on Y-axis. Data is from a representative experiment of $n$=3. Error bars represent standard deviation.

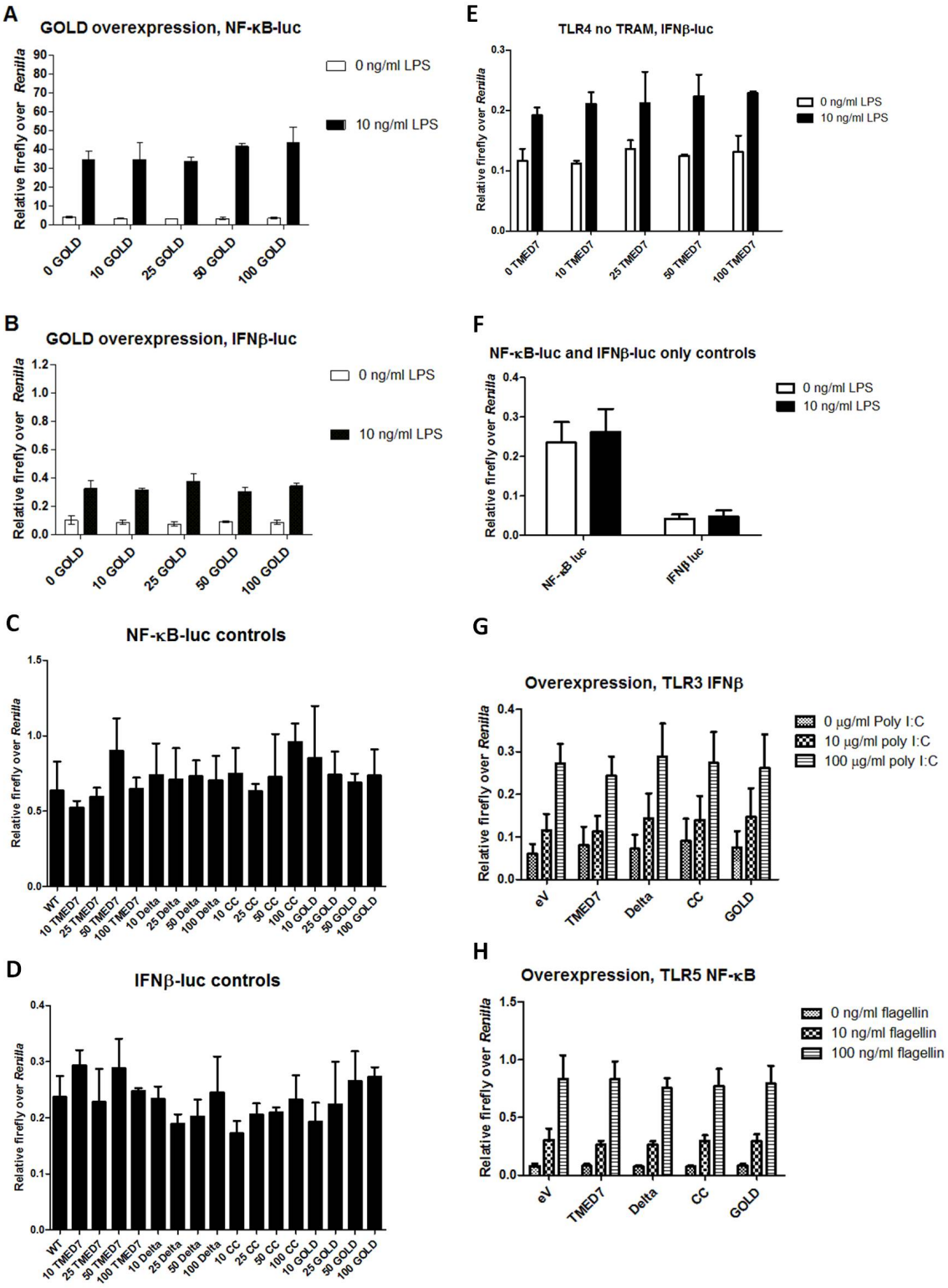
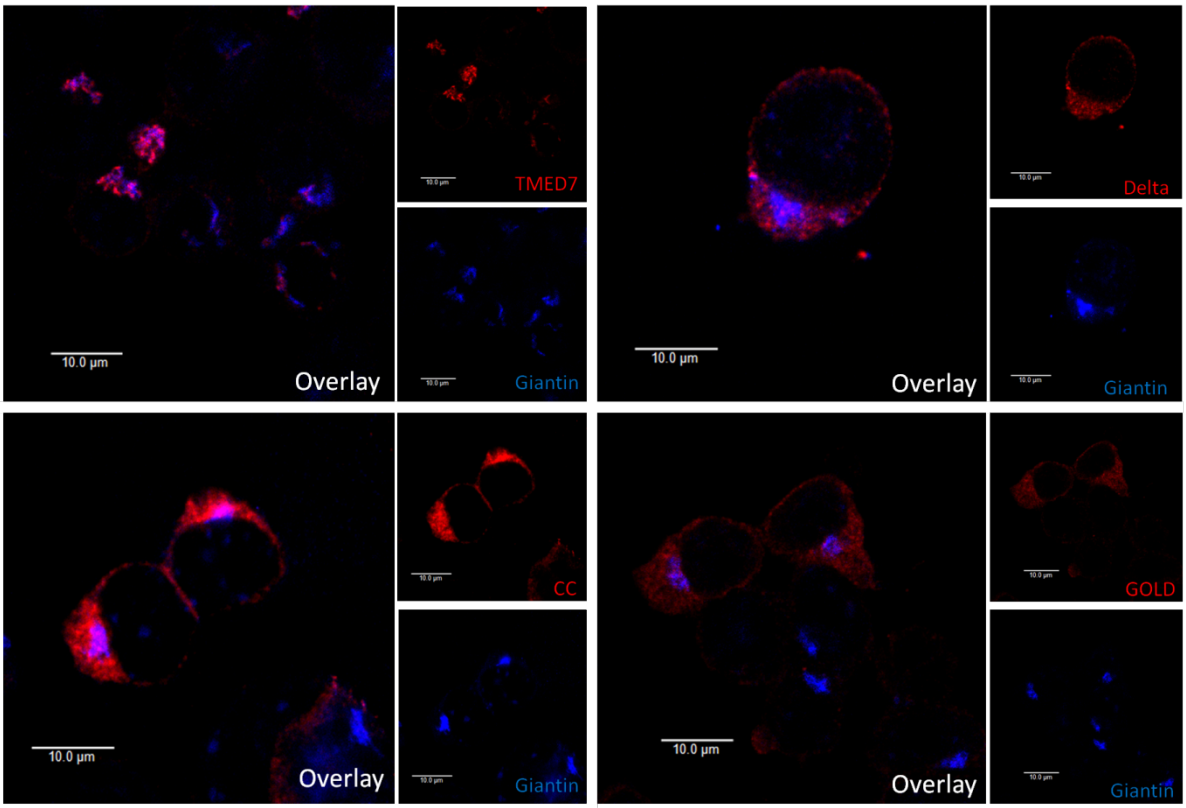



**Fig. S6. Control experiments of the luciferase reporter assays in HEK293 cells to ensure the specificity of the effect of TMED7 on TLR4 signaling.** This figure is related to Figures 5 and 6. GOLD-HA was used as a negative control in the reporter assay. HEK293 cells were transfected with TLR4, MD2, CD14, phRG, increasing amount of GOLD-HA, and NF-κB luciferase (**A**) or TRAM-FLAG and IFNβ luciferase (**B**) and cells were stimulated with LPS for 6 hours. (**C** and **D**) Controls to ensure that the effects of the overexpression observed were specific to TLR4. HEK293 cells were transfected with phRG and either NF-κB luciferase (**C**)

or IFNβ luciferase (**D**), with increasing plasmids indicated on the X-axis. Two days post transfection, luciferase activities were measured. (**E**) TRAM is required for IFNβ luciferase activation by TMED7. Cells were transfected as in (**B**) without TRAM-FLAG plasmid, and with increasing amount of TMED7-HA and stimulated with LPS for 6 hours. (**F**) HEK293 cells were transfected with phRG and either NF-κB luciferase or IFNβ luciferase and stimulated with LPS for 6 hours. (**G**) HEK293 cells were transfected with TLR3-FLAG, phRG, IFNβ luciferase, and the plasmid indicated on X-axis and stimulated with poly I:C for 6 hours. (**H**) HEK293 cells were transfected with phRG, NF-κB luciferase, and plasmids indicated on the X-axis and stimulated with flagellin for 6 hours. Data is presented as firefly luciferase activity normalized against *Renilla* luciferase activity on Y-axis. Data is from a representative experiment of $n$=3. Error bars represent standard deviation.

A


10.0 µm
Overlay
TMED7
Giantin
10.0 µm
Overlay
Delta
Giantin
10.0 µm
Overlay
CC
Giantin
10.0 µm
Overlay
GOLD
Giantin

B

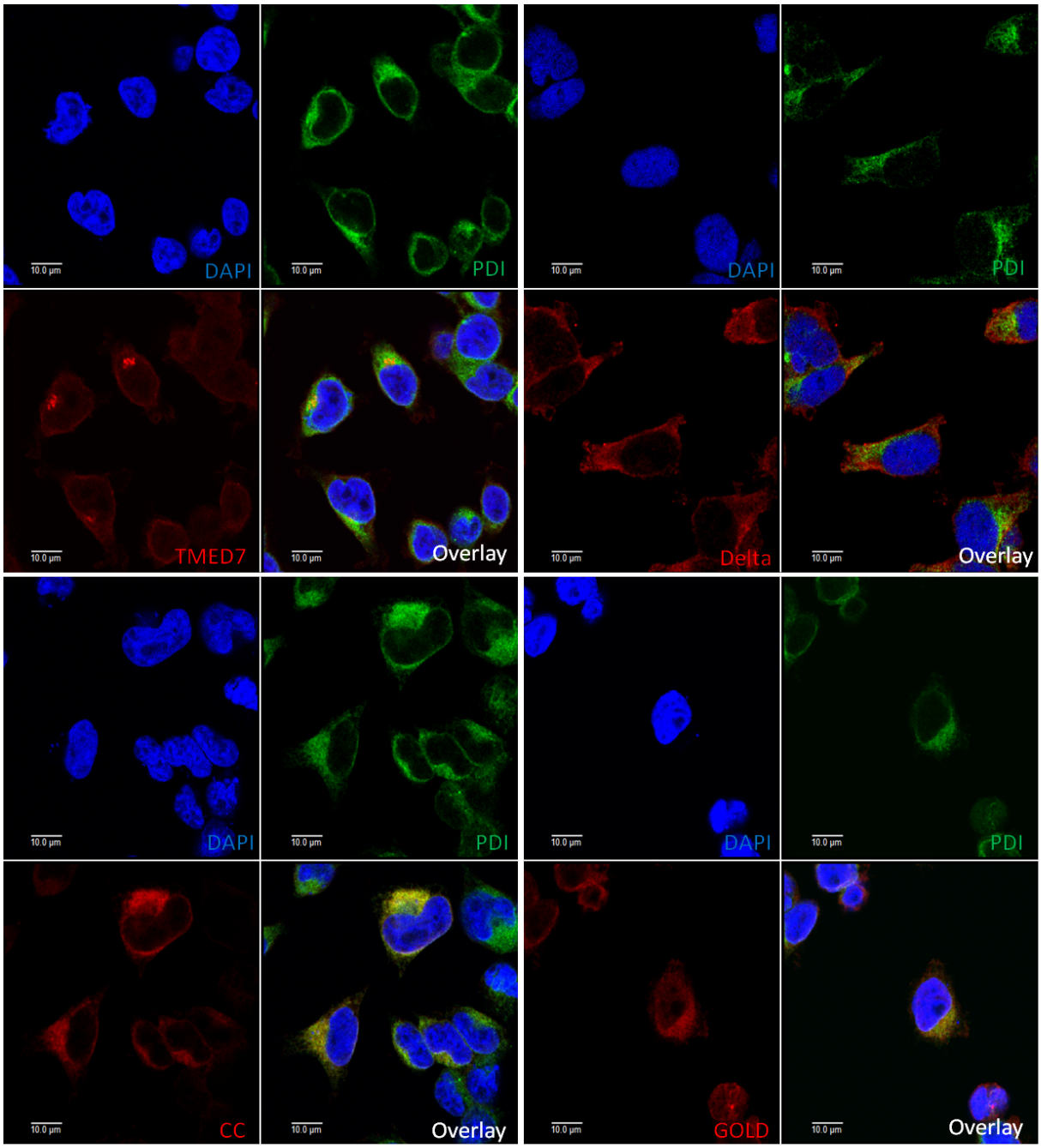



**Fig. S7. Golgi localization of TMED7 in HEK293T cells due to its cytosolic tail.** HEK293T cells were transiently transfected with HA-tagged TMED7, Delta, CC, or GOLD and incubated for 48 hours. Cells were then fixed, permeabilized, and immunofluorescently labelled to detect HA-tagged proteins (red) and Giantin (blue in A) or PDI (green in B). In (B) the nuclei were stained in DAPI. Approximately 20 cells were observed to have similar staining pattern in each condition from two independent experiments. Scale bar is 10 µm.

**Table S1. Primers for Gateway cloning for TMED7 CC, and GOLD with C-terminal FLAG or HA tag.** Underlined sequence is attB1 site; italicized sequences are attB2 sites. Sequences in bold are either start site (ATG) or stop site (CTA), followed by either TMED7 gene-specific sequence, or FLAG and TMED7 gene-specific sequence, or HA and TMED7 gene-specific sequence.

| | |
|---|---|
| TMED7 Fw | <u>GGGGACAAGTTTGTACAAAAAAGCAGGCT</u>TCACC**ATG**GGCCCGCG GCCGGGGTCCGCGCAG |
| TMED7 FLAG Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**CTTATCGTCGTCATC CTTGTAATCTGATCCAACACGAGTTGT |
| TMED7 HA Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**AGCGTAGTCTGGGA CGTCGTATGGGTATGATCCAACACGAGTTGT |
| CC FLAG Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**CTTATCGTCGTCATC CTTGTAATCGGCCACTCTTGTATTTAG |
| CC HA Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**AGCGTAGTCTGGGA CGTCGTATGGGTAGGCCACTCTTGTATTTAG |
| GOLD FLAG Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**CTTATCGTCGTCATC CTTGTAATCAAGAGCACTGACTCGGTT |
| GOLD HA Rv | *GGGGACCACTTTGTACAAGAAAGCTGGGTC***CTA**AGCGTAGTCTGGGA CGTCGTATGGGTAAAGAGCACTGACTCGGTT |

**Table S2. Primers for Quikchange mutagenesis to generate Delta mutant of TMED7 with C-terminal HA tag.**

| | |
|---|---|
| Delta Fw | CAGGTATTTCTTTTGAAAAGCTACCCATACGACGTCCCAGACTACGCT |
| Delta Rv | AGCGTAGTCTGGGACGTCGTATGGGTAGCTTTTCAAAAGAAATACCTG |

**Table S3. Amount of DNA plasmid transfected into HEK293 cells using jetPEI reagent for luciferase gene reporter assay.** Amounts shown here are per well in a 96-well plate.

For TLR4 NF-κB luciferase

| | |
|---|---|
| TLR4 | 10 ng |
| CD14 | 10 ng |
| MD2 | 1 ng |
| NF-κB-luc | 10 ng |
| phRG | 5 ng |
| HA-tagged TMED7, Delta, CC, or GOLD | 0, 1, 2, 5, 10 ng |
| pcDNA3.1 | to make a total of 100 ng |

For TLR4 IFNβ luciferase

| | |
|---|---|
| TLR4 | 10 ng |
| CD14 | 10 ng |
| MD2 | 1 ng |
| TRAM-FLAG | 2 ng |
| p125:IFNβ-luc | 20 ng |
| phRG | 5 ng |
| HA-tagged TMED7, Δ, CC, or GOLD | 0, 1, 2, 5, 10 ng |
| pcDNA3.1 | to make a total of 100 ng |

**Table S4. Amount of DNA plasmid transfected into HEK293T cells using jetPEI reagent for confocal microscopy and co-immunoprecipitation experiments.** Amounts shown here are per well in a 6-well plate.

| | |
|---|---|
| TLR4-citrine | 1 µg |
| CD14 | 500 ng |
| MD2 | 100 ng |
| pcDNA3: TRAM | 200 ng |
| HA-tagged TMED7, Delta, CC, and GOLD | 500 ng |
| pcDNA3.1 | to make a total of 3 µg |

**Table S5. Amount of DNA plasmid transfected into HEK293T cells using jetPEI reagent for flow cytometry experiments. Amounts shown here are per well in a 6-well plate.**

| | |
|---|---|
| FLAG-TLR4 | 1µg |
| MD2 | 100ng |
| HA-tagged TMED7, Delta, CC, and GOLD | 1µg |
| pcDNA3.1 | to make a total of 3µg |

**Table S6. Sequences of siRNA duplexes used in RNAi experiments.**

| | |
|---|---|
| siRNA95 sense | GUCAGUAGGAGAAGCCCUCAUUCUU |
| siRNA95 anti-sense | AAGAAUGAGGGCUUCUCCUACUGAC |
| siRNA2 sense | AAGCUUUUUCUCAGAUAAAUU |
| siRNA2 anti-sense | UUUAUCUGAGAAAAAGCUUUU |
| siRNA3 sense | GCUUUUUCUCAGAUAAAAGUU |
| siRNA3 anti-sense | CUUUUAUCUGAGAAAAAGCUU |

**Table S7. Sequences of gene-specific primers used in conventional and quantitative PCR.**

| | |
|---|---|
| GAPDH Fw | GAAGGTGAAGGTCGGAGTC |
| GAPDH Rv | GAAGATGGTGATGGGATTTC |
| TMED7 Fw | GCTCTTACCCAGATGGAATCT |
| TMED7 Rv | AGTTGTGGTGGTTCTTTTATC |
| TRAM Fw | TTCCTGCCCTCTTTCTCTCTC |
| TRAM Rv | AACATCTCTTCCACGCTCTGA |