The intracellular signaling pathway by which tumor necrosis factor (TNF) induces its pleiotropic actions is well characterized and includes unique components as well as modules shared with other signaling pathways. In addition to the currently known key effectors, further molecules may however modulate the biological response to TNF. In our attempt to characterize novel regulators of the TNF signaling cascade, we have identified transmembrane protein 9B (TMEM9B, c11orf15) as an important component of TNF signaling and a module shared with the interleukin 1β (IL-1β) and Toll-like receptor (TLR) pathways. TMEM9B is a glycosylated protein localized in membranes of the lysosome and partially in early endosomes. The expression of TMEM9B is required for the production of proinflammatory cytokines induced by TNF, IL-1β, and TLR ligands but not for apoptotic cell death triggered by TNF or Fas ligand. TMEM9B is essential in TNF activation of both the NF-κB and MAPK pathways. It acts downstream of RIP1 and upstream of the MAPK and IκB kinases at the level of the TAK1 complex. These findings indicate that TMEM9B is a key component of inflammatory signaling pathways and suggest that endosomal or lysosomal compartments regulate these pathways.

The two TNF receptors, TNFR1 (p55, TNFRSF1A) and TNFR2 (p75, TNFRSF1B), show high homology in their extracellular domains but less in their intracellular domains. Although soluble TNF binds TNFR1 with higher affinity than TNFR2 and therefore acts primarily via TNFR1, membrane-bound TNF activates equally TNFR1 and TNFR2 (3). In most tissues TNF signaling is mediated by TNFR1, whereas TNFR2 is restricted to fewer specific tissues, mostly of an immunological nature (3). Upon ligand binding, TNFR1 trimersizes and recruits TNF receptor-associated death domain protein (TRADD), receptor-interacting protein 1 (RIP1), and TNF receptor-associated factor 2 (TRAF2). This first complex acts as a platform at the plasma membrane to activate the NF-κB and MAPK signaling cascades, promoting cell survival and the expression of inflammatory cytokines. In a second step, TNFR1 is internalized into endocytic vesicles together with TRADD and RIP1 and recruits the proapoptotic molecules Fas-associated death domain (FADD) and caspase-8. This complex will initiate the apoptotic cell death program if concurrent anti-apoptotic NF-κB activation is absent (4).

The TGFβ-activated kinase 1 (TAK1) complex has a central function in many inflammatory pathways such as the TNFR, interleukin 1β receptor (IL-1R), and several Toll-like receptors (TLRs) (5, 6). This complex contains the catalytic subunit TAK1 and the adaptors TAK1-binding proteins 1, 2, and 3 (TAB1, TAB2, TAB3). Upon TNF stimulation, this complex interacts with TRAF2 leading to ubiquitin-mediated activation of TAK1. TAK1, in turn, phosphorylates and activates IκB kinase α and β (IKKα and IKKβ) and the MAPK kinases (MKK) (7). IKK phosphorylates the NF-κB inhibitor IκB leading to its degradation and to the subsequent release and nuclear translocation of NF-κB. In addition, phosphorylation of MKK4/7 and MKK3/6 by TAK1 leads to the activation of JNK (c-Jun N-terminal kinase) and of the p38 MAPKs. Activation of NF-κB and MAPK then coordinately promotes the expression of a wide variety of genes such as IL-6 and IL-8 that contribute to the proinflammatory role of TNF (2, 8).

Although the understanding of the TNF signaling cascade has advanced, many of the molecular mechanisms that regulate this pathway remain unclear. Here, we report the identification and characterization of a novel regulator of the TNF pathway, transmembrane protein 9B (TMEM9B, c11orf15). This molecule has been discovered previously as an NF-κB inducer in large scale cDNA overexpression screens (9). The function of TMEM9B and of its closest homolog, TMEM9 (10), is not known. Our results indicate that TMEM9B is a lysosomal transmembrane protein that regulates cytokine production induced by TNF via a novel signaling pathway.
not only by TNF but also by IL-1β and several TLR ligands by acting on the shared components RIP1 and TAK1. In contrast, TMEM9B is not involved in proapoptotic signaling of TNFR1 or Fas. Thus, TMEM9B is a new type of regulator of inflammatory signaling cascades.

EXPERIMENTAL PROCEDURES

Reagents, Plasmids, and siRNAs—The plasmids pCMV6XL5-TMEM9B and pCMV6XL5-RIPK1 were purchased from OriGene and pNF-xB-Luc and pRL-TK from Clontech and Promega, respectively. The cDNA of TMEM9B was subcloned into pEGFP-N1 (Clontech) by PCR. All other cDNA were expressed from plasmids constructed in pcDNA3.1 (Invitrogen). The pGL3-IL-8 promoter reporter plasmid contains nucleotides 2240–3765 of the human IL-8 promoter (clone AF385628). All siRNAs were synthesized at Novartis. Synthesis of siRNA was performed with standard phosphorodimide chemistry. All siRNAs are 21 oligonucleotides in length with a 19-base pair ribonucleotide duplex region and a two-deoxynucleotide overhang on the 3’-terminus of each strand. The 5’-ends of both strands are unphosphorylated. The following target sequences were used: GAA GCU CUG UCA CAA UCA A (TMEM9B siRNA1), GCA UGU UGU CCU CAG CUA A (TMEM9B siRNA2), CGG CAU UAU UGG AGU GAA A UCA A (TMEM9B siRNA3), GCA UGU UGU CCU CAG CUA A (p65), and CUU ACG CUG AGU ACU TCG A (luciferase). Recombinant human TNF and IL-1β were produced at Novartis. Synthesis of siRNA was performed with standard phosphorodimide chemistry. All siRNAs are 21 oligonucleotides in length with a 19-base pair ribonucleotide duplex region and a two-deoxynucleotide overhang on the 3’-terminus of each strand. The 5’-ends of both strands are unphosphorylated. The following target sequences were used: GAA GCU CUG UCA CAA UCA A (TMEM9B siRNA1), GCA UGU UGU CCU CAG CUA A (TMEM9B siRNA2), CGG CAU UAU UGG AGU GAA A UCA A (TMEM9B siRNA3), GCA UGU UGU CCU CAG CUA A (p65), and CUU ACG CUG AGU ACU TCG A (luciferase). Recombinant human TNF and IL-1β were produced at Novartis. TNF was biotinylated overnight at 4 °C with NHS-PEO4-Biotin (Pierce) in a phosphate buffer at pH 6.6 and dialyzed with PBS. The biological activity of biotin-TNF was identical to TNF with regard to IL-6 and IL-8 induction in HeLa cells (data not shown). Tunicamycin and LPS were from Sigma, super-Fas ligand from Alexis, Pam3Cys-SKKK (Pam3Cys) from EMC Microcollections, and poly(I-C) from GE Healthcare. The following antibodies were used: anti-TNFFR1 (H-5), anti-glyceraldehyde-3-phosphate dehydrogenase (Ambion, 6C5). Phospho-specific antibodies anti-MKK4, anti-MKK6, anti-IKKβ, and anti-p38 were from Cell Signaling. The antimouse IgG Alexa Fluor 488 and anti-rabbit IgG Alexa Fluor 633 were from Invitrogen. The TMEM9B antibody was produced and purified at Eurogentec by immunizing rabbits with the peptide CQEQRKSVFDRHVVLSC coupled to keyhole limpet hemocyanin (KLH).

Cell Culture and Transfection—The cell lines 293-EBNA, ME180 (both from Invitrogen), and HeLa (from ATCC) were maintained in DMEM (Invitrogen) supplemented with 10% fetal calf serum. Normal human dermal fibroblasts from neonatal skin (Lonza) were cultured in FGM-2 (Lonza) for less than 10 passages. Unless indicated otherwise, cells were stimulated with TNF at 30 ng/ml, IL-1β at 10 ng/ml, LPS at 10 ng/ml, poly(I-C) at 0.5 µg/ml, and Pam3Cys at 1 µg/ml. For siRNA transfection, HeLa, ME180, or primary human dermal fibroblasts were seeded in 150 µl of culture medium in 96-well plates. Then 50 µl of Opti-MEM (Invitrogen) containing 20 µM siRNA and 0.75 µl of HiPerFect (Qiagen) were added to the cells. For transfection of 293-EBNA with DNA, cells were seeded in 100 µl of culture medium, and then 10 µl of Opti-MEM containing 0.8 µl of FuGENE (Roche Applied Science) and 75 µg of luciferase reporter plasmid, 5 ng of pRL-TK, and 120 ng of expression plasmid were added to the cells. For sequential transfection of HeLa cells with siRNA and DNA, cells were seeded in 2 ml of culture medium in 6-well plates and then 100 µl of Opti-MEM containing 120 nm siRNA and 6 µl of HiPerFect were added to the cells. Two days later, cells were trypsinized and replated in a 96-well plate. Four hours later, the culture medium was replaced by 100 µl of Opti-MEM containing 100 ng of plasmid DNA and 1 µl of GenePORTER (Genlanis). Transfection medium was replaced three hours later by DMEM, 2% fetal calf serum.

Immunoblotting, Pulldown, and Subcellular Fractionation—For analysis of phosphoproteins, cells were stimulated with TNF for the indicated amount of time, washed once with ice-cold PBS, and lysed in 50 mM Tris, pH 7.4, 5 mM EDTA, 1% SDS.
Lysates were heated at 95 °C for 5 min and sonicated, and protein concentration was determined with a bichinchoninic acid assay (Sigma). Equal amounts of proteins were resolved on 4–12% polyacrylamide NuPAGE gels (Invitrogen) and analyzed by immunoblotting. For isolation of the TNFR1 complex, HeLa cells were incubated with cold DMEM containing 100 ng/ml biotinylated TNF for 10 min. Excess biotinyl-TNF was washed off with ice-cold PBS, and then the cells were incubated with prewarmed DMEM at 37 °C for the indicated time and rinsed with ice-cold PBS. Cells were lysed in lysis buffer A containing 20 mM Tris pH 7.4, 150 mM NaCl, 1% Triton X-100, 10 mM β-glycerol-phosphate, 1 mM EDTA, 1 mM EGTA, 10 mM NaF, and protease inhibitors (Roche Applied Science). After being precleared with protein G-agarose (Roche Applied Science), lysates were incubated with streptavidin-agarose beads (Sigma) for 3 h. Beads were washed five times with lysis buffer, and bound proteins were analyzed by immunoblot as described above. Nuclei were isolated using NE-PER reagent, and integral and transmembrane proteins were isolated by phase partitioning using the Mem-PER reagent as recommended by the manufacturer (Pierce).

Cell Surface Biotinylation—HeLa cells were incubated with 0.2 mg/ml NHS-PEO4-Biotin (Pierce) in PBS for 30 min at 4 °C under gentle rocking. Cells were washed with cold PBS, incubated for 30 min at 4 °C with DMEM to quench unreacted biotin, and washed again with cold PBS. Cells were then lysed in lysis buffer A and incubated with streptavidin-agarose beads for 2 h. Bound proteins were analyzed by immunoblot as described above.

IL-6, IL-8, Cell Viability, and Reporter Gene Assay—The concentrations of IL-6 or IL-8 were determined in 10 μl of cell culture supernatant by HTRF® assays as recommended by the manufacturer (Cisbio). Cell viability was assessed with ATPlute 1Step (PerkinElmer Life Sciences). Promoter activity of IL-8 and NF-κB reporter plasmids in 293-EBNA cells was assayed with a dual-luciferase reporter assay system (Promega). The ratio of firefly luciferase activity to Renilla luciferase activity was used to normalize for transfection variability.

Quantitative PCR—For the determination of IL-6 and IL-8 mRNA levels, total RNA was isolated on RNeasy micro-columns (Qiagen), and cDNA was synthesized using Superscript III (Invitrogen). On aliquots of this cDNA, quantitative real-time PCR was performed simultaneously with primers and probes (TaqMan Assays-on-Demand) for S18 ribosomal RNA (VIC-labeled) and for IL-6 or IL-8 (both FAM-labeled) with the TaqMan Fast Universal PCR mix (all from Applied Biosystems). IL-6 and IL-8 mRNA levels were normalized to S18 rRNA levels.

Confocal Microscopy—HeLa cells stably expressing a TMEM9B-EGFP fusion protein were grown on coverslips and fixed with 4% formaldehyde in PBS. Fixed cells were permeabilized in 0.1% saponin, 3% horse serum in PBS and then stained sequentially with primary and secondary antibodies coupled to Alexa Fluor 488 and Alexa Fluor 633, respectively. Nuclei were stained blue with 4,6-diamidino-2-phenylindole.

RESULTS

TMEM9B Is a Glycosylated Lysosomal Transmembrane Protein—The amino acid sequence of TMEM9B has been shown to contain an N-terminal signal peptide at positions 1–33 (11). Cleavage of the signal peptide gives rise to mature TMEM9B (molecular mass, 19.0 kDa), which is predicted to be glycosylated at Asn-60. Incubation of HeLa cells with tunicamycin, an inhibitor of N-glycosylation, led to a decrease of the apparent molecular mass of TMEM9B from about 22 to 19 kDa (Fig. 1A), confirming that TMEM9B is an N-glycosylated protein.

Sequence analysis indicated that TMEM9B may contain a transmembrane helix located in the signal peptide at position 7–29 and a second transmembrane domain at position 103–125 (TMHMM server v. 2.0 (12)). To determine whether TMEM9B is a membrane spanning protein, HeLa cell proteins were fractionated by phase partitioning and analyzed by immunoblot. A strong enrichment of TMEM9B in the hydrophobic fraction mainly containing membrane proteins could be detected, suggesting that TMEM9B is a membrane protein (Fig. 1B). To determine whether TMEM9B is localized at the plasma...
membrane and is accessible at the outer membrane leaflet, cell surface proteins were biotinylated. HeLa cells were incubated with amine-reactive hydrophilic biotin, and biotinylated proteins were isolated on streptavidin beads. In contrast to the positive control TNFR1, TMEM9B could not be detected in the bound fraction (Fig. 1C), indicating that TMEM9B is not accessible at the outer plasma membrane but that it is an intracellular membrane protein. To assess whether TMEM9B is a secreted protein, tagged TMEM9B was transiently overexpressed in HeLa cells, and after 24 h supernatant was analyzed by immunoprecipitation for the presence of soluble tagged TMEM9B. No secreted TMEM9B could be detected (data not shown).

To investigate its subcellular localization, a stable HeLa cell clone expressing TMEM9B fused to EGFP was analyzed. No TMEM9B-EGFP could be detected at the plasma membrane of live cells by confocal microscopy (Fig. 2A), confirming the result of the cell surface biotinylation assay. A distinct perinuclear and vesicular structure was however revealed by the TMEM9B-EGFP fluorescence (Fig. 2A). To determine the nature of this structure, double staining with markers for lysosomes, early endosomes, and the Golgi apparatus was performed in fixed cells. Fig. 2B shows strong co-localization (in yellow) of TMEM9B with the lysosomal marker LAMP1 and partial colocalization with the early endosomal marker, EEA1. In contrast, no colocalization with the Golgi protein mannosidase could be observed. Taken together, these data show that TMEM9B is a transmembrane protein localized primarily in the lysosome and, to a limited extent, in early endosomes.

Function of TMEM9B in the TNF Signaling Cascade—The two proinflammatory cytokines IL-6 and IL-8 are induced by TNF in many different cell types. To determine whether TMEM9B is involved in the TNF signaling cascade, we first assessed whether the expression of TMEM9B was necessary for the production of IL-6 and IL-8 in HeLa cells. Endogenous TMEM9B was depleted with specific siRNA, and IL-6 and IL-8 were measured in the cell culture supernatant. Two different siRNAs against TMEM9B reduced the production of IL-6 induced by TNF stimulation by 61 and 35% and of IL-8 by 47 and 24% (Fig. 3A). The effect of the siRNAs on cytokine secretion correlated with an efficient reduction of TMEM9B protein expression (Fig. 3B).

The role of TMEM9B in the TNF signaling cascade was further confirmed in primary human dermal fibroblasts. Similarly to HeLa cells, an siRNA against TMEM9B inhibited the production of IL-8 induced by TNF by 62% (Fig. 4A) with a concomitant reduction of TMEM9B protein levels (Fig. 4B). We next determined whether TMEM9B was involved in other inflammatory signaling cascades in primary human dermal fibroblasts. Depletion of TMEM9B reduced IL-1β-induced IL-8 by 56% and the IL-8 production induced by ligands of TLR2 (Pam3Cys), TLR3 (poly(I:C)), and TLR4 (LPS) by 58, 68, and 77%, respectively (Fig. 4A). These data show that TMEM9B is required for the production of proinflammatory cytokines not only downstream of the TNFR1 but also downstream of the IL-1 receptor and several TLRs, suggesting that TMEM9B regulates a common proinflammatory signaling module.

TMEM9B Is Required for Cytokine Production in Response to Several Inflammatory Stimuli—In addition to promote the expression of proinflammatory cytokines, TNF also triggers apoptotic cell death. We sought therefore to determine whether TMEM9B regulated TNF-induced apoptosis. The ME180 cell line is sensitive to TNF-induced apoptosis (13), and treatment with TNF resulted in about 20 and 30% dead cells (after 10 and 100 ng/ml TNF, respectively; Fig. 5A). Cell death was TNF-specific, as it was blocked by an siRNA against TNFR1. In contrast, reduction of TMEM9B expression in ME180 cells with an siRNA (Fig. 5B) did not significantly inhibit cell death compared with cells transfected with the control siRNA.

FIGURE 3. TMEM9B is required for TNF-induced cytokine expression in HeLa cells. A. HeLa cells were transfected with two different siRNAs against TMEM9B, an siRNA against TNFR1, or a control siRNA against luciferase and stimulated 2 days later with TNF. IL-6 and IL-8 concentrations in cell culture supernatants were determined and normalized to the control siRNA and cell viability. The averages ± S.D. of four independent experiments are shown; *, indicates statistical significance (p < 0.01 compared with control siRNA). B, siRNA-transfected HeLa cells were lysed, and TMEM9B expression in cell lysates was analyzed by immunoblot using an anti-TMEM9B antibody. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

FIGURE 4. TMEM9B is involved in multiple inflammatory signaling cascades in primary human dermal fibroblasts. A, human dermal fibroblast were transfected with an siRNA against TMEM9B or a control siRNA and stimulated 2 days later with TNF or IL-1β for 8 h or with LPS, poly(I:C), or Pam3Cys for 16 h. The IL-8 concentration in cell culture supernatants was determined and normalized to cells transfected with the control siRNA. The averages ± S.D. of three independent experiments are shown; *, indicates statistical significance (p < 0.01 compared with control siRNA). B, the TMEM9B levels of siRNA-transfected dermal fibroblasts were analyzed by immunoblot. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
The HeLa cell line is not sensitive to TNF-induced apoptosis, but it is sensitive to Fas ligand, a related member of the TNF family. Stimulation with 200 ng/ml hexameric super-Fas ligand killed about 50% of the cells after 16 h (Fig. 5C). Knockdown of TMEM9B did not affect Fas ligand-induced cell death. Together, these data demonstrate that the induction of apoptosis by the TNF family members TNF and Fas ligand is independent of TMEM9B and that TMEM9B is specifically involved in inflammatory cytokine signaling.

FIGURE 5. TMEM9B is not required for TNF- or Fas ligand-induced apoptosis. ME180 (A and B) or HeLa cells (C) were transfected with an siRNA against TMEM9B or TNFR1 or a control siRNA. One day later, cells were stimulated with TNF (A) or super-Fas ligand (sFasL) (C). After 18 h, cell viability was determined. The averages ± S.D. of at least three independent experiments are shown (n.s., not significant). TMEM9B levels in siRNA-transfected ME180 were analyzed by immunoblot (B). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

TMEM9B Regulates Cytokine Expression at the Transcriptional Level—The reduction in cytokine production after TMEM9B depletion could result from a defect in gene transcription, mRNA translation, or protein secretion. We therefore assessed whether cytokine mRNA levels were affected by TMEM9B. The IL-6 and IL-8 mRNA levels of HeLa cells transfected with TMEM9B siRNA were determined by quantitative PCR. One representative experiment is shown. B, 293-EBNA cells were transfected with increasing amounts of a TMEM9B expression vector and an IL-8 promoter luciferase reporter vector. One day later, cells were stimulated with TNF or left unstimulated for 4 h before luciferase activity was determined. Data are the average of three independent experiments.

FIGURE 6. TMEM9B modulates TNF-induced cytokine expression at the transcriptional level. A, HeLa cells were transfected with a siRNA against TMEM9B or a control siRNA. Two days later, cells were stimulated for the indicated time, RNA was isolated, and IL-6 and IL-8 mRNA levels were determined by quantitative PCR. One representative experiment is shown.  

B, 293-EBNA cells were transfected with increasing amounts of a TMEM9B expression vector and an IL-8 promoter luciferase reporter vector. One day later, cells were stimulated with TNF or left unstimulated for 4 h before luciferase activity was determined. Data are the average of three independent experiments.
TMEM9B on IL-8 promoter activity was determined by cotransfection of a TMEM9B expression vector and an IL-8 promoter reporter. Overexpression of TMEM9B activated the IL-8 promoter in a dose-dependent manner to a level similar to that observed upon TNF stimulation (Fig. 6B). Together, these data show that TMEM9B is both necessary and sufficient for TNF-induced cytokine gene transcription.

**TMEM9B Acts Downstream of the TNFR1 Complex and of RIP1**—Upon ligand binding, TNFR1 recruits TRADD and RIP1 to form a complex that is internalized into signaling vesicles termed TNF receptosomes (14). To determine whether TMEM9B is recruited to TNF receptosomes, we isolated the active TNFR1 complex using biotinylated TNF. Incubation of HeLa cells with biotinyl-TNF lead to the recruitment of TRADD as well as of polyubiquitinated RIP1 (Fig. 7A). Because TMEM9B could not be detected in the biotinyl-TNF pulldown, it is likely not recruited to the TNFR1 complex but probably acts further downstream of the TNFR1 complex.

Polyubiquitinated RIP1 has been shown to serve as a platform to recruit the TAK1 and IKK complexes, leading to the activation of the IKKs by assessing the phosphorylation state of IKKα/β (15). We sought to determine whether TMEM9B acts upstream or downstream of the IKKs by assessing the phosphorylation state of IKKα/β (Fig. 8A) without affecting the expression level of IKKα and IKKβ (Fig. 8F). This indicates that TMEM9B modulates the NF-κB pathway by acting upstream of the IKKs.

**TMEM9B Is Required for Activation of the JNK and p38 MAPK Signaling Cascades**—In addition to activating the NF-κB pathway, TNF also triggers the p38 and JNK signaling cascades via RIP1 and TAK1. In HeLa cells TNF induced the phosphorylation of the two upstream kinases MKK3 and MKK6 and of p38 and MK2, a p38 substrate. An siRNA against TMEM9B almost completely abrogated phosphorylation of MKK3/6 and MK2 (Fig. 8D) but did not affect the total amount of MKK4 or p38 (Fig. 8F). Similarly, the phosphorylation but not the total amount of MKK4, as well as the phosphorylation of its substrate, and the two JNK isoforms p46 and p54 were strongly reduced (Fig. 8E and F). These data show that in addition to its function in the activation of the NF-κB pathway, TMEM9B is also required for the activation of the p38 MAPK and JNK signaling cascades, positioning TMEM9B in a proximal and common position of proinflammatory cytokine signaling pathways.

**DISCUSSION**

TNF activates a complex signaling network that leads to two major outcomes: cell activation and apoptosis (4). In our attempt to better characterize the TNFR1 cascade, we uncovered a function of TMEM9B as an essential component of several inflammatory pathways. We show that TMEM9B is a lysosomal membrane protein involved in the proinflammatory but not in the apoptotic arm of the TNF signaling cascade at the
level of TAK1. The expression of TMEM9B was required for the production of IL-6 and IL-8 induced not only by TNF but also immune stimuli like IL-1β and several TLR ligands. Depletion of TMEM9B resulted in reduced transcriptional activation of the IL-8 promoter via NF-κB and diminished TNF-mediated MAPK signaling.

The epistasis experiments performed in this study suggest that TMEM9B acts at the level of TAK1. Indeed, the knockdown of TMEM9B inhibited the cytokine induction by RIP1 or TAK1, indicating interference downstream of RIP1 and TAK1. Because reduction of TMEM9B expression also resulted in a reduction of the phosphorylation of the TAK1 substrates IKKα/β, MKK3/6, and MKK4, TMEM9B very likely acts at the level of TAK1.

Further evidence for the role of TMEM9B in TAK1 regulation is that TMEM9B is involved not only in the TNF signaling cascade but also downstream of the IL-1R and several TLRs, mirroring the central role of TAK1 in the very same pathways (17, 18). The molecules required for the activation of TAK1 differ between these receptors. Whereas TNFR1 signals via TRAF2 and RIP1, the IL-1R and TLRs require MYD88, IRAK4, and TRAF6 (5, 19). Precisely how TMEM9B controls TAK1 is unclear; TMEM9B may be required either for the phosphorylation and ubiquitin-mediated activation of TAK1 or for the recruitment of TAK1 substrates to the TAK1-TAB complex.

We observed that TMEM9B is localized to vesicular structures of the lysosome. The lack of adequate reagents precluded an analysis of the subcellular localization of endogenous TMEM9B, but a stable low expressing clone of a TMEM9B-EGFP fusion could be used. With this caveat, our data on TMEM9B are reminiscent of the subcellular localization of TMEM9, the closest homolog of TMEM9B, which can be found in lysosomes and late endosomes when overexpressed in COS cells (10). The function of TMEM9, however, is unknown, although a role in intracellular transport was suggested.

The localization of TMEM9B in lysosomes and early endosomes suggests that these organelles are involved in the regulation of signal transduction downstream of inflammatory receptors. Interestingly, the activation of mitogen-activated protein kinase kinase 1 (MEK1) and its downstream substrate, extracellular-signal regulated protein kinase 1/2 (ERK1/2), by epidermal growth factor requires localization of MEK1 and ERK1/2 to the endosome via the endosomal protein p14. Binding of p14 to the scaffold protein MEK partner 1 (MP1) recruits MEK1 (20). It has been shown recently that p14 deficiency abrogates late endosomal biogenesis and ERK1/2 activation, leading to a new type of human primary immunodeficiency (21). Our data showing the involvement of a lysosomal protein in the activation of the NF-κB and MAPK pathways suggest by analogy that the lysosomal and/or endosomal compartments may play a central role also in inflammatory signaling. One might speculate that TMEM9B, like p14, could act as an anchor for a signaling complex upstream of NF-κB and p38. Interestingly, the TAK1-TAB complex is associated to a membrane structure in unstimulated cells and translocates to the cytosol upon IL-1β stimulation (22). Whether the TAK1-TAB complex associates with the lysosomal membrane and whether this is mediated by TMEM9B are currently under investigation. A direct interac-

**FIGURE 8. TMEM9B is required for NF-κB and MAPK activation and acts upstream of IKK and MKKs.** A, 293-EBNA cells were transfected with an NF-κB promoter luciferase reporter vector and increasing amounts of a TMEM9B expression vector. One day later, luciferase activity was determined. Data are the average of three independent experiments. **B**, HeLa cells were transfected with a siRNA against TMEM9B or a control siRNA and were stimulated 2 days later with TNF for 30 min. Nuclear proteins were isolated and analyzed for p65 levels. C, HeLa cells were transfected with an siRNA against TMEM9B or a control siRNA and were stimulated 2 days later for the indicated time. Cells were lysed, and phosphorylation of p65 and IKK was assessed by immunoblot. D and E, HeLa cells were transfected with an siRNA against TMEM9B or a control siRNA and were stimulated 2 days later for the indicated time. Cells were lysed, and phosphorylation of MKK3, MKK6, MKK4, p38, MK2, and JNK was assessed by immunoblot. F, HeLa cells were transfected with an siRNA against TMEM9B or a control siRNA, and the expression level of the indicated proteins was assessed by immunoblot. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
tion between TMEM9B and TAK1 or any TAB could not be detected (data not shown), suggesting that TMEM9B may modulate TAK1 indirectly.

Our observations are compatible with the notion that a first membrane-proximal TNFR1 complex exists that signals to NF-κB and MAPK prior to an internalized TNFR1 receptosome which mediates apoptotic signaling (14, 23). Depletion of TMEM9B did not affect TNF or Fas ligand-induced apoptosis or surface TNFR1 binding to biotinyl-TNF (data not shown). In addition, we did not detect a direct interaction between the TNFR1 complex and TMEM9B. Therefore, our data suggest that TMEM9B is required for the activation of a TAK1 signaling complex that is downstream of the membrane-proximal TNFR1 complex and receptosome and is shared with several inflammatory signals.

In summary, we have described TMEM9B as a glycosylated membrane protein located in the lysosome, demonstrating that TMEM9B is required for the activation of the NF-κB and MAPK pathways by inflammatory stimuli. This is the first report to characterize TMEM9B function and also to indicate the relevance of a lysosomal protein in the signaling cascades of inflammatory receptors.

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