

A DAP12-mediated Pathway Regulates Expression of CC Chemokine Receptor 7 and Maturation of Human Dendritic Cells

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Abstract

Gene targeting of the adaptor molecule DAP12 in mice caused abnormal distribution and impaired antigen presentation capacity of dendritic cells (DCs). However, the DAP12-associated receptors expressed on DCs and their functions have not been identified yet. Here we show that the triggering receptor expressed on myeloid cells-2 (TREM-2) is a cell surface receptor on human monocyte-derived DCs, which is associated with DAP12. TREM-2/DAP12 promotes upregulation of CC chemokine receptor 7, partial DC maturation, and DC survival through activation of protein tyrosine kinases and extracellular signal-regulated kinase. In contrast to Toll-like receptor-mediated signaling, TREM2/DAP12 stimulation is independent of nuclear factor- κ B and p38 stress-activated protein kinase. This novel DC activation pathway may regulate DC homeostasis and amplify DC responses to pathogens, explaining the phenotype observed in DAP12-deficient mice.

Key words: TREM • activation • human • survival • migration

Introduction

Dendritic cells (DCs)* are a distinct population of bone marrow-derived leukocytes that initiate primary and secondary immune responses (1). DCs migrate from the blood to peripheral tissues, where they reside in an immature state, awaiting antigen encounter. Upon antigen capture, DCs process them into peptides, which are loaded onto MHC molecules for presentation to T cells. As a result of pathogen invasion, inflammation, and tissue damage, DCs receive additional activating signals, which induce a profound change in DC phenotype and functions, known as maturation (1–3). Mature DCs express the chemokine receptor CCR7, which interacts with the chemokines CCL19 (also

known as EBI-1 ligand chemokine [ELC], or macrophage inflammatory protein 3 β [MIP-3 β]) and CCL21 (also known as secondary lymphoid tissue chemokine [SLC], or 6-C-Kine) (4–9). These chemokines are crucial for guiding DCs from peripheral tissues to draining lymph nodes, as demonstrated in mice with natural or targeted genetic deletions of CCL19, CCL21, or CCR7 (10–14). In addition, mature DCs express high levels of stable MHC-peptide complexes on the cell surface, upregulate costimulatory and adhesion molecules, and downregulate antigen-capturing molecules. Thus, mature DCs can efficiently present antigens and stimulate virgin-T cells located in the T cell-rich areas of lymph nodes (1, 15). Here, DCs receive further activating signals from cognate Th cells, which express CD40 ligand (CD40L) (16), OX40 (17, 18), and TNF-related activation-induced cytokine (TRANCE) (19–22). These stimuli trigger IL-12 secretion by antigen presenting DCs thus promoting Th1 type T cell responses (20, 23–27).

Activating signals induce DC maturation through several distinct signaling pathways. LPS, other bacterial and viral components, as well as products released by damaged tissues, activate DCs through Toll-like receptors (TLRs) (28, 29). TLRs trigger downstream signaling pathways, which activate the nuclear factor (NF)- κ B. NF- κ B promotes the transcription of a variety of genes mediating maturation (30–33). In addition, TLRs activate mitogen-activated pro-

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*Abbreviations used in this paper: CCR7, CC chemokine receptor 7; DC, dendritic cell; EMSA, electrophoretic mobility assay; ERK, extracellular signal-regulated kinase; HRP, horseradish peroxidase; ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; MDL-1, myeloid DAP12-associating lectin-1; NF, nuclear factor; PTK, protein tyrosine kinase; SAPK, stress-activated protein kinase; SIRP- β , signal-regulatory protein β ; TLR, Toll-like receptor; TREM, triggering receptor expressed on myeloid cells.

tein kinases (MAPKs), such as the stress-activated protein kinase p38 (P38/SAPK) and the extracellular signal-regulated kinase (ERK), which concur to DC activation (28, 29). Inflammatory cytokines, such as IL-1, IL-18, and TNF- α , and T cell surface molecules, such as CD40L, OX40, and TRANCE, bind specific receptors that activate NF- κ B as well (1, 20, 34–37). A second pathway of DC maturation is initiated by the receptors for the Fc portion of IgG (FcRs), which bind antibody-opsonized pathogens (38). FcRs lack intracellular signaling motifs, but display a charged residue in the transmembrane domain, which mediates association with the γ chain of FcR (FcR γ) (39). FcR γ contains a cytoplasmic immunoreceptor tyrosine-based activation motif (ITAM), which recruits protein tyrosine kinases (PTKs) of the *src* and *syk* families. PTKs trigger protein tyrosine phosphorylation, Ca²⁺ mobilization, and phosphorylation of several MAPKs (40, 41). While FcR γ is essential for FcR-mediated DC maturation (42), the role of downstream PTKs and MAPKs is yet unknown.

Recent observations suggest that DC activation is controlled by yet another signaling pathway, which involves the adaptor molecule DAP12 (also called KARAP). DAP12 is associated with several NK and myeloid cells activating receptors (43–54). Like FcR γ , DAP12 contains a cytoplasmic ITAM, recruits the PTKs ZAP70 and p72/syk, and promotes activation of ERK (44, 45, 55, 56). Knock-in mice bearing a nonfunctional mutation within the ITAM of DAP12 showed a dramatic accumulation of DCs in mucocutaneous epithelia and were resistant to hapten-specific contact sensitivity (57). In addition, DAP12-deficient mice were resistant to experimental autoimmune encephalomyelitis (EAE) induced by immunization with myelin oligodendrocyte glycoprotein peptide (58). These phenotypes suggested a role of DAP12 in regulating migration and antigen presentation capacity of DCs. Three DAP12-associated receptors have been identified in myeloid cells. One of these, myeloid DAP12-associating lectin-1 (MDL-1), is a member of the C-type lectin superfamily (50). The others, signal-regulatory protein β (SIRP- β) and triggering receptor expressed on myeloid cells-1 (TREM-1), belong to the Ig superfamily (53, 59). TREM-1 is preferentially expressed on neutrophils and a subset of blood monocytes (53). SIRP- β and MDL-1 are mainly expressed on blood monocytes and macrophages (50, 60). When monocytes are differentiated toward DCs by culturing them in vitro in the presence of GM-CSF and IL-4, expression of MDL-1, SIRP- β , and TREM-1 is completely downregulated (50, 53, 60).

Recently, we have cloned a cell surface receptor distantly related to TREM-1 called TREM-2. TREM-2 is a member of the Ig-superfamily characterized by a single V-type extracellular domain, a transmembrane region with a charged residue of lysine and a short cytoplasmic tail with no signaling motifs (53). Here we found that TREM-2 is associated with DAP12 and, in contrast to TREM-1, SIRP- β , and MDL-1, is not expressed on monocytes, but it is strongly upregulated on human DCs derived in vitro from monocytes. This observation provided the opportu-

nity to investigate the role of TREM2/DAP12-mediated signaling pathways in DC migration and maturation.

Materials and Methods

Production of TREM-2 Human IgM Fusion Protein. Soluble TREM-2 was produced as a chimeric protein consisting of TREM-2 extracellular domain and human IgM constant regions (TREM-2 human IgM [TREM-2-HuIgM]), as previously described (61). TREM-2 extracellular domain was amplified from the cloned full length cDNA by polymerase chain reaction using the following oligonucleotides: 5'-ACTCTGCTTCTGCCCT-TGGCTGGGG, 3'-tagtagGTCGACATACTTACCGGGTGG-GAAAGGGATTTCTCCTTCCAA. Purification of TREM-2-HuIgM from culture supernatants was performed by affinity chromatography on Sepharose-coupled mouse anti-human IgM mAb (Sigma-Aldrich) according to manufacturer's protocols.

Transfections. 293 cells were transiently transfected with a cDNA encoding human TREM-2 as a FLAG peptide NH₂-terminal fusion protein (Eastman Kodak Co.) using cytofectene (Bio-Rad Laboratories).

Production and Modifications of Anti-TREM-2 and Control mAbs. 6-wk-old BALB/c mice (Iffa-Credo) were immunized with purified TREM-2-HuIgM. Spleen cells were fused with the SP2/0 myeloma cells and hybridoma supernatants were screened by ELISA using TREM-2-HuIgM as capturing protein and human-adsorbed horseradish peroxidase (HRP)-labeled goat anti-mouse IgG (BD PharMingen) as detecting Ab. ELISA-positive hybridoma supernatants were then tested by flow cytometry for staining 293 cells expressing FLAG-tagged TREM-2. mAb 29E3 (anti-TREM-2, IgG1, κ), mAb 21C7 (control IgG1, κ , anti-TREM-1) (53), and mAb 1B7.11 (control IgG1, κ , anti-2,4,6 TNP; American Type Culture Collection) were purified using GammaBind-Sepharose (Amersham Pharmacia Biotech). Purified mAbs were either biotinylated (Roche) or labeled with Cy5 (Amersham Pharmacia Biotech) according to manufacturer's protocols. F(ab') or F(ab')₂ fragments of mAb 29E3 and mAb 21C7 were prepared using the F(ab')/F(ab')₂ Kit (Pierce Chemical Co.). F(ab') and F(ab')₂ were separated from the Fc portion by affinity chromatography on protein G-sepharose, followed by gel filtration on a Superdex 75 HR10/30 (Amersham Pharmacia Biotech). F(ab') and F(ab')₂ preparations were tested for the absence of Fc fragments by immunoassay. F(ab') and F(ab')₂ fragments were biotinylated allowing for crosslinking by ExtrAvidine (Sigma-Aldrich) or flow cytometry by Streptavidin-allophycocyanin (APC) or -PE (BD PharMingen). Alternatively, F(ab')₂ fragments were crosslinked using a goat anti-mouse IgG F(ab')₂ specific antibody (The Jackson Laboratory).

Cells. PBMCs were purified from human blood by gradient density centrifugation on lymphocyte separation medium (LSM; ICN Biomedicals/Cappel). CD14⁺ monocytes were purified from PBMCs by magnetic cell sorting (MACS) using CD14 MicroBeads (Miltenyi Biotec). Monocyte-derived DCs were prepared from purified monocytes as described previously (62, 63).

Antibodies and Flow Cytometry. Before staining, all cells were preincubated with PBS-20% human serum for 1 h on ice to block Fc receptors (FcR). Monocytes cultured in M-CSF or GM-CSF and IL-4 were stained with either mAb 29E3, mAb 21C7, or mAb 1B7.11, followed by human-adsorbed PE-conjugated goat anti-mouse IgG (Southern Biotechnology Associates, Inc.). In three-color stainings, immature DCs cultured with LPS (100 ng/ml), TNF- α (10 ng/ml), or CD40L-transfected J558L cells (64) were incubated with Cy5-labeled mAbs 29E3 and FITC-conju-

gated anti-CD83 mAb (Immunotech). Cells were analyzed on a FACSCalibur™ cytometer using CELLQuest™ software (Becton Dickinson). Dead cells were excluded by gating on PI-negative cells.

Stimulation of DCs by LPS, F(ab')₂ Anti-TREM-2 mAb, or Human IgG in the Presence or Absence of Inhibitors. Human IgG, F(ab')₂ 29E3 (anti-TREM-2 mAb), or control F(ab')₂ (21C7 anti-TREM-1 mAb) were coated for 6 h at 37°C on 96-well flat-bottom plates with a final concentration of 20 µg/ml in PBS. LPS was used at a final concentration of 1 µg/ml. Immature DCs were plated at a concentration of 5 × 10⁵ cells/well and simultaneous contact to the plate was induced by short centrifugation (400 g, 1 min, 25°C). Supernatants and cells were collected after 6, 12, 24, 36, 48, or 72 h and tested by ELISA or flow cytometry. In blocking experiments, inhibitors (PD98059 [20 µM], LY294002 [10 µM], SB203580 [2 µM], PP2 [1 µM]; all from Calbiochem), and TPCK (20 µM; Sigma-Aldrich) were added 60 min before stimulation.

Measurement of Cytokines, Chemokines, and Cell Surface Activation Markers. To measure stimulation-dependent changes in the expression of cell surface markers and cytokine secretion, monocyte-derived DCs were stimulated as described above for 6, 12, 24, 48, and 72 h. Supernatants were collected and tested for production of IL-6, IL-8, IL-10, TGF-β, IL-12p40, IL-12p75, IL-13, IL-15, IL-18, IL-1α, IL-1β, TNF-α, and MCP-1 by ELISA (BD PharMingen). Cells were harvested and stained with anti-TREM-2, -MHC class I, -MHC class II, -CD1a, -CD11a, -CD11b, -CD11c, -CD29, -CD32, -CD35, CD38-, CD40-, -CD41, -CD54, -CD61, -CD64, -CD80, -CD83, -CD86, -CD89, -CD103, -CD115, -CD116, -CCR5, -CCR6, -CXCR4, or -Mannose receptor conjugated with Cy5-, PE-, or FITC (all from Immunotech and BD PharMingen). Anti-CCR7 mAb (BD PharMingen) was followed by F(ab')₂ PE-labeled goat anti-mouse IgM Ab (Southern Biotechnology Associates, Inc.). Stained cells were analyzed by flow cytometry.

Measurement of Cytosolic Ca²⁺. Monocyte-derived DCs were loaded with Indo-1 AM (Sigma-Aldrich) for 30 min at 37°C, washed three times, and resuspended in RPMI/10 mM HEPES/5% FCS. Cytoplasmic Ca²⁺ levels were monitored in individual cells by measuring 405/525 spectral emission ratio of loaded Indo-1 dye by flow cytometry. After a baseline was acquired for at least 30 s, 29E3, 21C7, F(ab')₂ 29E3, F(ab')₂ 21C7, F(ab')₂ 29E3, or F(ab')₂ 21C7 were added to a final concentration of 1 µg/ml and analysis was continued up to 512 s. All antibodies and antibody fragments were biotinylated. In some experiments, ExtraAvidine (Sigma-Aldrich) was added as crosslinker together with the biotinylated primary antibodies or Ab fragments.

Determination of ERK, JNK, and p38/SAPK Activation. Monocyte-derived DCs (10⁶ cells per time point) were stimulated as described above. After 0 (unstimulated control), 1, 2, 5, 10, and 20 min cells were harvested and chilled on ice. Cells were spun down and lysed in reducing sample buffer. Specific induction of tyrosine phosphorylation and phosphorylation of ERK, p38/SAPK, and JNK was determined by reducing Western blot analysis using anti-phospho-ERK, anti-ERK, anti-phospho-p38/SAPK, anti-p38/SAPK, anti-phospho-JNK, and anti-JNK antibodies (all from New England Biolabs, Inc.).

Surface Biotinylation and Pervanadate Treatment. Monocyte-derived DCs were washed three times in PBS followed by incubation with sulfo-NHS-biotin according to the manufacturer's protocol (Pierce Chemical Co.). For pervanadate treatment, cells were incubated with 200 µM pervanadate and 200 µM H₂O₂ at 37°C for 5 min. Biotinylation or pervanadate stimulation was stopped by wash-

ing the cells three times in PBS/10% FCS/200 µM pervanadate and one time with ice cold PBS/200 µM pervanadate, respectively.

Immunoprecipitations. Surface-biotinylated cells were lysed in 1% digitonin, 100 mM Tris-HCl, pH 7.4, 150 mM NaCl, protease inhibitors (Complete; Roche Molecular Biochemicals). After overnight preclearing with normal mouse serum coupled to protein G Sepharose 4B (Amersham Pharmacia Biotech), lysates were subjected to immunoprecipitation with 5 µg/ml of 29E3, 21C7, or 1B7.11 at 4°C for 3 h. Immunocomplexes were precipitated by addition of protein-G-Sepharose 4B for 3 h at 4°C. Precipitates were washed four times with lysis buffer, followed by a final wash with 0.5% digitonin, 100 mM Tris-HCl, pH7.4, 150 mM NaCl. After separation by SDS-PAGE, precipitates were analyzed by Western blot with HRP-conjugated streptavidin. In deglycosylation experiments the precipitates were incubated for 18 h with or without N-Glycanase F (Roche) according to the manufacturer's protocol. Pervanadate-treated cells were subjected to immunoprecipitation as described above. Immunoprecipitates were analyzed by Western blot with antiphosphotyrosine PY20-HRP (Transduction Laboratories) or anti-DAP12 rabbit antiserum followed by human/mouse-adsorbed anti-rabbit IgG-HRP (Southern Biotechnology Associates, Inc.).

Chemotaxis Assay. Monocyte-derived human DCs (10⁷) were treated for 24 h with F(ab')₂ 21C7, F(ab')₂ 29E3 coated on plastic (20 µg/ml), or LPS (1 µg/ml). Cells (5 × 10⁵ in 100 µl IMDM/0.5% BSA) were incubated for 1 h at 37°C. Cells were subsequently loaded into collagen-coated Transwells (Costar; 3-µm pore filter), which were placed onto 24-well plates containing 450 µl medium supplement with 100 ng/ml CCL19 (ELC/MIP-3β) or CCL20 (6-C-Kine/SLC) (both from Pepro-Tech). After an incubation period of 4 h at 37°C, cells that had migrated to the lower chamber were collected and counted on a cytofluorimeter (FACSCalibur™, constant time acquisition; Becton Dickinson). In blocking experiments cells were preincubated with anti-CCR7 mAb (10 µg/ml) and added to the Transwell.

Detection of Apoptosis. Determination of DNA fragmentation was performed as described previously (65). Inhibitors of kinases or serine proteases (PD98059 [20 µM], LY294002 [10 µM], TPCK [20 µM]) were added 60 min before stimulation. Inhibitors had no effect on cell viability or the rate of constitutive apoptosis at the indicated concentrations.

Nuclear Extracts and Electrophoretic Mobility Shift Assays. Nuclear extracts were prepared according to the method of Schreiber et al. (66) with some modifications. Stimulation of monocyte-derived human DCs (10⁷) with control or anti-TREM-2 antibody or with LPS was performed for 0.5 or 4 h at 37°C as described above. Cells were washed in PBS, resuspended in 10 ml of ice-cold buffer A (10 mM Tris-HCl, pH 7.9, 60 mM KCl, 1 mM EDTA, 0.75 mM spermidine, 0.15 mM spermine, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, and 1 µg/ml pepstatin), and incubated for 15 min on ice. Nonidet P-40 was added from a 10% stock solution to a final concentration of 0.6%, and samples were vortexed for 10 s. After incubation for 3 min on ice, samples were centrifuged at 3,000 rpm for 10 min at 4°C. Nuclei were washed in 10 ml of ice-cold buffer A and resuspended in 30 ml of ice-cold buffer C (20 mM Tris-HCl, pH 8, 0.4 M NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 0.5 mM PMSF, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 25% glycerol). Nuclei were incubated for 30 min at 4°C, and nuclear extracts were separated from debris by centrifugation at 15,000 g for 15 min at 4°C. Protein concentrations were determined by Bradford assay using Bio-Rad protein assay (Bio-Rad Laboratories). NF-κB consensus and mutant binding sites

were 5'-AGTTGAGGGGACTTCCAGGC-3' and 5'-AGT-TGAGGCGACTTTCCAGGC-3', respectively. Annealed binding sites were radiolabeled using polynucleotide T4 kinase and γ [³²P]-ATP. Radiolabeled oligonucleotides were purified by electrophoresis through an 8% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA, overnight elution from gel slices at 37°C, concentration using Elutip-d columns (Schleicher & Schuell), and ethanol precipitation. Electrophoretic mobility assays (EMSA) were performed as described previously (67) with some modifications. Nuclear extracts (2 μ g) were incubated with 1 μ g of poly(dI-dC) carrier and 1 μ g of BSA in a 25 μ l reaction mix containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 1 mM EDTA, and 5% glycerol for 10 min at 4°C in the presence or absence of 25-fold excess of unlabeled oligonucleotide

competitors. Labeled binding-site probes (15 fmols, $\sim 5 \times 10^4$ cpm) were then added for an additional 20 min of incubation at 4°C. Samples were electrophoresed through a 4% polyacrylamide gel containing 22.5 mM Tris-borate and 0.5 mM EDTA at 4°C.

Results

Human Immature Monocyte-derived DCs Express TREM-2, a ~ 40 kD Glycoprotein which Is Associated with DAP12. In initial studies, TREM-2 transcript was selectively detected in monocyte-derived DCs by reverse transcriptase (RT)-PCR (data not shown). To precisely investigate the cellular distribution of TREM-2 as well as its biochemical characteristics and functions, we produced an anti-TREM-2 mAb (29E3). This antibody stained TREM-2-transfected 293 cells specifically, compared with control transfectants (Fig. 1 A). In agreement with RT-PCR data, TREM-2 was highly expressed on DCs derived from peripheral blood monocytes upon in vitro culture with GM-CSF and IL-4 (Fig. 1 B). DC maturation induced by LPS, TNF α , CD40L-expressing cells (Fig. 1 C), IL-1 β , CpG oligonucleotides, or aggregated IgG (data not shown) led to complete downregulation of TREM-2. TREM-2 was undetectable on macrophages obtained by culturing monocytes up to 14 d with M-CSF (Fig. 1 B) and on primary DCs of peripheral blood (data not shown). Thus, TREM-2 is preferentially expressed on immature monocyte-derived DCs.

Immunoprecipitation of TREM-2 from surface-biotinylated monocyte-derived DCs revealed that TREM-2 is a glycoprotein of ~ 40 kD, that is reduced to 26 kD after N-deglycosylation (Fig. 2 A). This result is in agreement with the predicted molecular mass of TREM-2 (53). As TREM-2 lacks known signaling motifs in the cytoplasmic domain and displays a charged residue of lysine in the transmembrane domain (53), it was likely to be associated with a separate adaptor molecule to transduce signals. Adaptor molecules, such as DAP12, DAP10, and FcR γ are tyrosine phosphorylated upon cell treatment with the phosphatase inhibitor pervanadate (44, 68, 69). Indeed, anti-phosphoty-

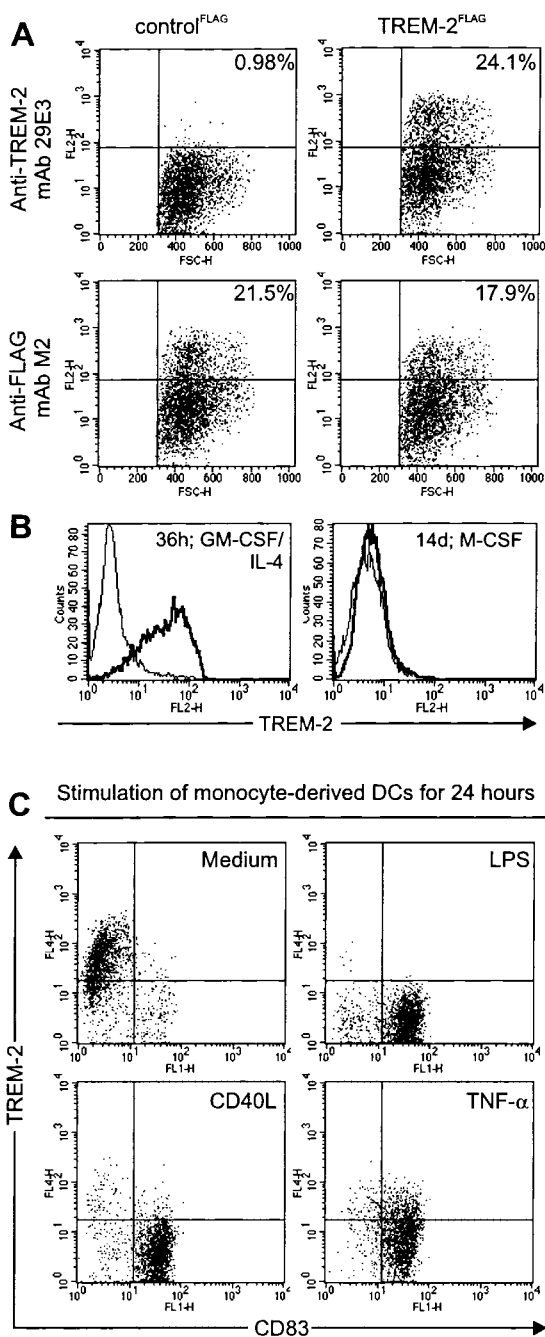


Figure 1. TREM-2 is selectively expressed on immature DCs. (A) mAb 29E3 recognizes selectively TREM-2. 293 cells transfected with a cDNA encoding FLAG-tagged TREM-2 (TREM-2^{FLAG}; right panels) were stained with mAb 29E3 (top panel), compared with cells transfected with a control cDNA (control^{FLAG}; left panels). The percentages of positive cells (top right quadrants) are indicated. Expression of TREM-2^{FLAG} and control^{FLAG} was confirmed using an anti-FLAG mAb (bottom panels). Cells stained with an isotype-matched control mAbs were comprised within the indicated bottom quadrant. (B) TREM-2 is strongly upregulated after stimulation of monocytes with GM-CSF and IL-4. Monocytes treated with GM-CSF/IL-4 (left panel) or M-CSF (right panel) were analyzed by flow cytometry for cell surface expression of TREM-2 (solid bold line) after 36 h or up to 14 d, respectively. Dashed profiles indicate background staining with a control IgG₁ mAb. (C) TREM-2 is rapidly downregulated upon maturation of DCs. LPS- (top right panel), CD40L- (bottom left panel), TNF α -stimulated (bottom right panel), or unstimulated monocyte-derived DCs (top left panel) were analyzed by flow cytometry for cell surface expression of TREM-2 and CD83 after 36 h. Cells stained with an isotype-matched control mAbs were comprised within the indicated bottom quadrants.

rosine blotting of TREM-2 immunoprecipitates from per-vanadate-stimulated monocyte-derived DCs revealed a phosphorylated protein of ~14 and ~28 kD under reducing and nonreducing conditions, respectively (Fig. 2 B). This pattern was consistent with the association of TREM-2 with a tyrosine-phosphorylated protein that forms a disulfide-linked homodimer. Immunoblotting of TREM-2 immunoprecipitates with anti-DAP12, -DAP10, and -FcR γ antisera demonstrated that TREM-2, like TREM-1, associates only with DAP12 (Fig. 2 C, and data not shown). Thus, TREM-2 is capable of stimulating DAP12-linked signaling pathways in DCs.

TREM-2 Induces ERK Activation and Survival of DCs. To see whether the TREM-2/DAP12 complex transduces activating signals in DCs as other DAP12-associated receptors do in NK cells and neutrophils (43–45, 47–54, 56, 70), we stimulated TREM-2 with 29E3 mAb or with its F(ab')₂ fragment. In both cases, ligation of TREM-2 elicited a rapid rise in intracellular Ca²⁺ concentration of DCs (Fig. 3 B). However, monovalent engagement of TREM-2 using F(ab') 29E3 did not induce calcium mobilization, indicating that TREM-2-mediated activation needs at least two or more receptors crosslinked (data not shown). Cross-linking of TREM-2 with F(ab')₂ 29E3 stimulated tyrosine phosphorylation of several proteins with approximate molecular masses of ~110, ~90, ~60–70, and ~30–40 kD (Fig. 3 C). The observed ~40 kD tyrosine phosphorylated proteins corresponded to the ERK1/2, as demonstrated by antiphospho-ERK1/2 immunoblotting (Fig. 3 D). It was previously shown that survival of LPS-stimulated DCs is dependent on ERK (71) and phosphatidylinositol 3-kinase (PI-3K) (72), while maturation is mainly mediated through NF- κ B. Therefore, we tested whether stimulation of TREM-2 leads to prolonged survival of DCs kept in culture in the absence of GM-CSF or IL-4. As shown in Fig. 3 E, crosslinking of TREM-2 with F(ab')₂ 29E3 prolonged DC survival for almost 8 d. Treatment of TREM-2-stimulated DCs with the ERK inhibitor PD98059 blocked this survival effect. Inhibitors of PI-3K, I κ B-phosphorylation, or I κ B-degradation had no effect (Fig. 3 F, and data not shown). These observations indicate that TREM-2 induces survival of DCs through activation of the ERK pathway.

TREM-2 Triggers Rapid Upregulation of CCR7 and Increased Expression of MHC Class II, CD86, and CD40. To examine whether TREM-2 can trigger migration of DCs and/or their maturation into efficient antigen-presenting cells, immature DCs were stimulated with F(ab')₂ 29E3 mAb coated on plastic surface. These cells were tested for the expression of cell surface molecules involved in migration, antigen presentation, costimulation, and adhesion, as well as for the production of cytokines. Upon TREM-2 ligation, CCR7 surface expression was rapidly increased (Fig. 4 A). CCR7 was functionally competent, as TREM-2-stimulated DCs showed a specific chemotactic response towards the CCL19 and CCL21, which could be inhibited by anti-CCR7 mAb (Fig. 4 B). The amplitude and kinetics of CCR7 upregulation induced via TREM-2 were different compared with those induced by

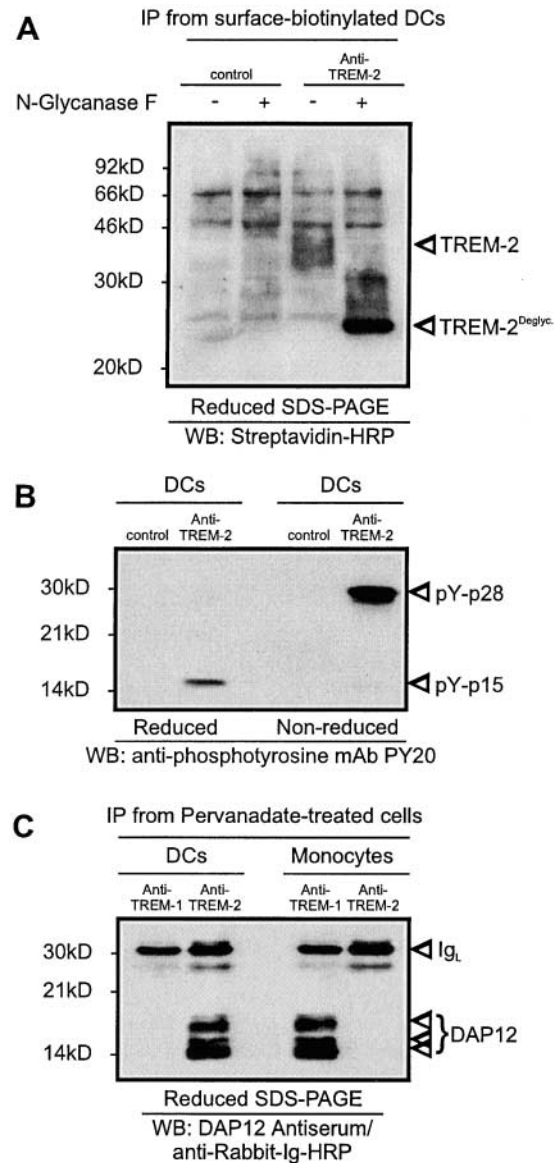


Figure 2. TREM-1 is a 40-kD glycoprotein associated with the adaptor protein DAP12. (A) Surface-biotinylated monocyte-derived DCs were lysed and subjected to immunoprecipitation with 29E3 anti-TREM-2 mAb (right lanes) or control IgG₁ mAbs (21C7 anti-TREM-1 mAb). Immunoprecipitates (IP) were left untreated or treated with N-glycanase F and analyzed by Western blot analysis with Streptavidine-HRP. Molecular weight markers and specific protein bands are indicated. (B) Pervanadate-treated monocyte-derived DCs were subjected to immunoprecipitation with 29E3 anti-TREM-2 mAb or control IgG₁ mAb (21C7 anti-TREM-1 mAb). The precipitates were analyzed by antiphosphotyrosine blot under reducing (left lanes) and nonreducing (right lanes) conditions. Tyrosine phosphorylated proteins are marked by arrows. Molecular weight markers are indicated. (C) Anti-DAP12 blot analysis of TREM-2 immunoprecipitate from monocyte-derived DCs (left lanes) and monocytes (right lanes) after pervanadate stimulation (reducing conditions). TREM-1 immunoprecipitates from monocytes and monocyte-derived DCs were included as positive and negative controls, respectively. Molecular weight markers and specific protein bands are indicated.

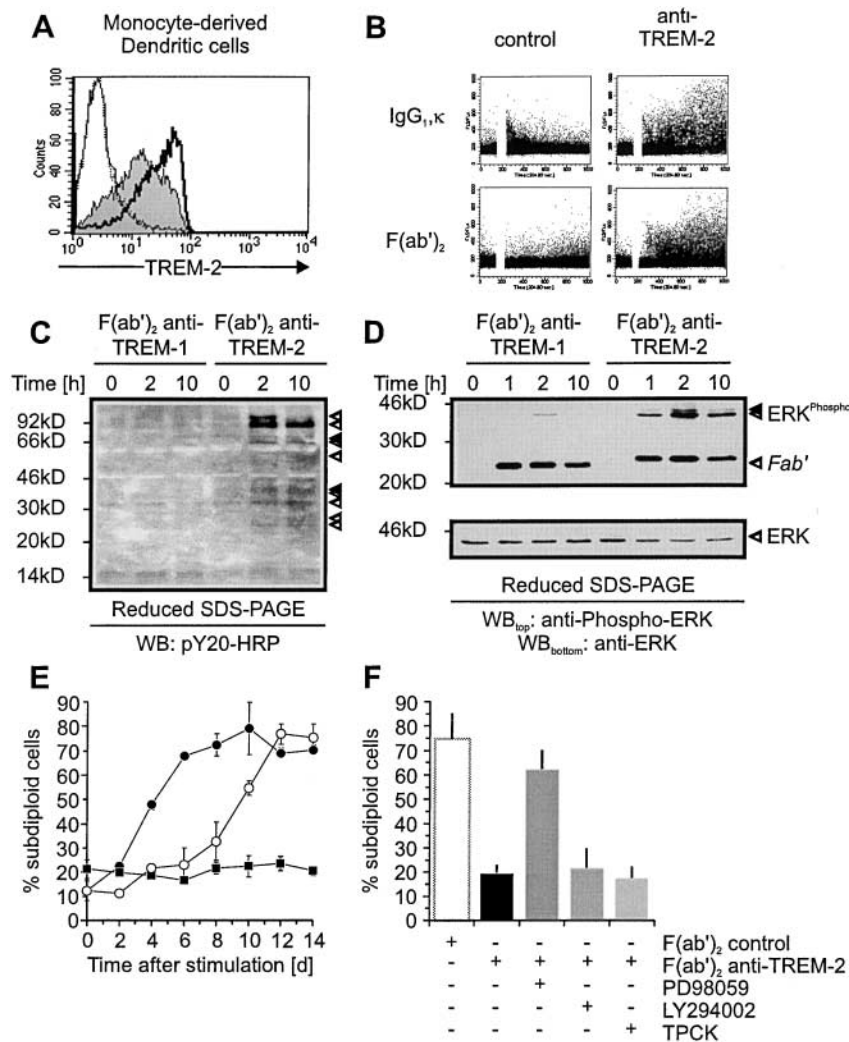


Figure 3. Stimulation of monocyte-derived DCs via TREM-2 induces calcium mobilization, tyrosine phosphorylation, and prolongs DC survival by an Erk-dependent pathway. (A) Functional characterization of F(ab') and F(ab')₂ 29E3. Monocyte-derived DCs were analyzed by flow cytometry for cell surface expression of TREM-2 using either biotinylated F(ab')₂ 29E3 (solid bold profile), F(ab') 29E3 (gray profile), control F(ab') (dashed profile), or F(ab')₂ (solid profile) followed by Streptavidin-PE. (B) Stimulation of TREM-2 induces intracellular Ca²⁺ mobilization. Bivalent crosslinking of TREM-2 using IgG1, κ 29E3, or Fc-free F(ab')₂ 29E3 induces intracellular Ca²⁺ mobilization in contrast to control mAbs (21C7 anti-TREM-1 mAb; left panels; 1A11 anti-MHC class I mAb; data not shown). (C) Antiphosphotyrosine blot of cell lysates from monocyte-derived DCs stimulated with F(ab')₂ 29E3 (anti-TREM-2) or control F(ab')₂ (anti-TREM-1 mAb) for the indicated time periods. (D) Monocyte-derived DCs were stimulated as indicated in C and examined by Western blot analysis for anti-phospho-Erk1/2 (top panel) compared with anti-Erk 1/2 (bottom panel). Arrows indicate phosphorylated proteins in all panels. Molecular weight markers are shown. (E) Monocyte-derived DCs were washed five times to remove GM-CSF/IL-4 before stimulation with plastic-bound F(ab')₂ 29E3 (open circles), control F(ab')₂ (21C7 anti-TREM-1 mAb) (filled circles), or GM-CSF (filled squares) for the indicated time periods. Apoptotic cell death was determined by measurement of DNA fragmentation. (F) Monocyte-derived DCs were stimulated plastic-bound F(ab')₂ 29E3 in the presence or absence of Erk-inhibition (PD98059 [20 μM]), PI3K-inhibition (LY294002 [10 μM]), or inhibition of IκB-degradation (TPCK [20 μM]). Apoptotic cell death was determined after 8 d compared with DCs stimulated with control F(ab')₂ by measuring subdiploid DNA content.

LPS stimulation (Fig. 4 A). While expression of CCR7 was detectable by 6 h after TREM-2 stimulation, LPS-induced upregulation of CCR7 was weaker and occurred only after 24 h of cell stimulation. Regardless of CCR7 surface levels, however, LPS-stimulated DCs displayed a stronger chemotactic response toward CCL19 and CCL21

than TREM-2-stimulated DCs. Therefore, DC mobility and chemotaxis to CCL19 and CCL21 is not solely related to CCR7 expression levels. Indeed, it has been shown that other receptors, such as the multidrug resistance-associated protein 1, can contribute to optimal mobilization of DCs from skin to secondary lymphoid organs (73).

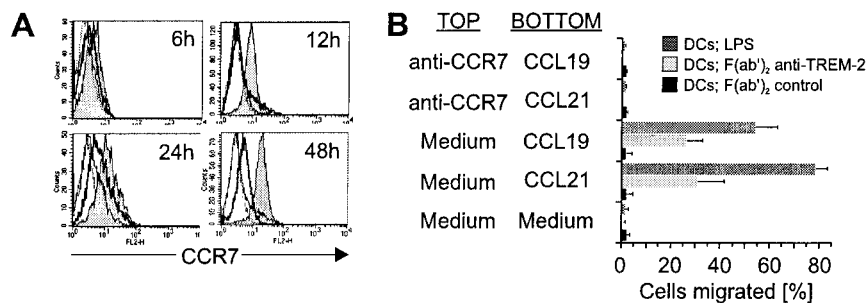


Figure 4. Stimulation of TREM-2 induces CCR7 expression and chemotactic response toward CCL19 and CCL21. (A) CCR7 is rapidly upregulated after stimulation of TREM-2 on DCs. DCs were stimulated with F(ab')₂ anti-TREM-2 mAb (gray profiles), control F(ab')₂ (21C7; anti-TREM-1; solid line profiles), or LPS (solid bold profiles). After the indicated time periods cells were harvested and analyzed by flow cytometry for cell surface expression of CCR7 by anti-CCR7 mAb (mouse IgM) followed by PE-labeled goat anti-mouse IgM. Dashed profiles indicate background staining.

with a control IgM mAb. (B) TREM-2 stimulation directs migration of DCs toward CCL19 and CCL21 by a CCR7-dependent pathway. DCs stimulated for 24 h with plastic-coated control F(ab')₂ (21C7; anti-TREM-1; black bars), F(ab')₂ anti-TREM-2 mAb (light gray bars), or LPS (dark gray bars) were tested in Transwell chemotaxis assays for the ability to migrate toward medium alone, medium supplemented with 100 ng/ml CCL19 or CCL21 (BOTTOM well). In control experiments, DCs were preincubated for 15 min with anti-CCR7 mAb before placing them in the TOP well for assessment of chemotaxis toward medium alone, medium supplemented with CCL19, or CCL21 placed in the BOTTOM well.

Table I. *TREM-2–dependent Regulation of Cell Surface Activation Markers*

	F(ab') ₂ anti-TREM-1	F(ab') ₂ anti-TREM-2	LPS
TREM-2	112.23	3.45	7.1
MHC class I	67.8	65.3	107.1
MHC class II	119.12	278.65	454.67
CD40	171.35	598.6	635.89
CD80	32.1	39.4	104.6
CD86/B7.2	14.04	387.91	683.56
CCR5	12.95	13.56	3.12
CCR6	3.68	3.45	4.01
CCR7	6.82	21.98	12.45
CXCR4	5.13	4.56	17.8
CD11a	10.92	6.78	13.72
CD11b	53.9	65.7	23.1
CD11c	91.1	65.7	123.5
CD29	38.22	37.56	37.5
CD41	4.54	4.67	4.39
CD54/ICAM-1	56.87	54.78	271.45
CD61	4.95	5.03	4.21
CD103	3.63	3.96	3.26
Mannose-R	81.8	82.9	30.9
CD64/FcγR I	9.8	10.1	2.3
CD32/FcγR II	17.21	16.78	2.34
CD89/FcαR	4.54	4.75	4.96
CD35/CR 1	3.94	4.23	3.67
M-CSF-R	14.6	4.23	5.21
GM-CSF-R	15.6	13.7	13.5
CD38	2.5	2.2	43.5
CD83	3.34	3.23	26.7
CD1a	106.76	134.9	87.54

DCs were cultured for 48 h in plates coated with control F(ab')₂, F(ab')₂ anti-TREM-2 mAb, or LPS as indicated in Fig. 6. Cells were subsequently analyzed by flow cytometry for the indicated cell surface molecules. Numerical values indicate specific mean fluorescence intensity after subtraction of the fluorescence detected with an isotype-matched control. The data shown are representative of four independent experiments.

Ligation of TREM-2 also induced increased cell surface expression of several molecules involved in T cell stimulation, such as MHC class II, CD40, and CD86 (Table I). In contrast to LPS-activated DCs, CD83 and intracellular adhesion molecule (ICAM)-1 were not upregulated. Further-

Table II. *Lack of Cytokine and Chemokine Secretion upon TREM-2 Engagement*

	F(ab') ₂ anti-TREM-1	F(ab') ₂ anti-TREM-2	LPS
IL-1α	N.D.	N.D.	0.135 ± 0.026
IL-1β	0.027 ± 0.012	N.D.	0.162 ± 0.09
TNF-α	0.042 ± 0.005	N.D.	4.015 ± 0.078
IL-18	N.D.	N.D.	2.56 ± 1.31
IL-6	N.D.	N.D.	16.7 ± 5.43
IL-10	N.D.	N.D.	2.03 ± 0.45
TGF-β1	N.D.	N.D.	N.D.
IL-12p40	N.D.	N.D.	3.48 ± 1.25
IL-12p70	N.D.	N.D.	1.45 ± 0.09
IL-13	N.D.	N.D.	N.D.
IL-15	N.D.	N.D.	N.D.
MCP-1	2.018 ± 0.875	0.449 ± 0.067	98.18 ± 35.86
IL-8	1.23 ± 0.451	0.023 ± 0.01	124.76 ± 23.91

Cells were stimulated as described in Fig. 6. Cell supernatants were analyzed by ELISA for secretion of the indicated cytokines and chemokines. Data are representative for three independent experiments. Values are shown in pg/ml. N.D., not detectable.

more, antigen-capturing molecules, such as CD32, CD64, CD89, and the mannose receptor, were not downregulated (Table I). DCs activated through TREM-2 did not secrete either IL-12, or other cytokines (Table II). Thus, TREM-2 mediates a unique pattern of DC activation, characterized by upregulation of CCR7, expression of some T cell stimulatory molecules, and lack of cytokine secretion.

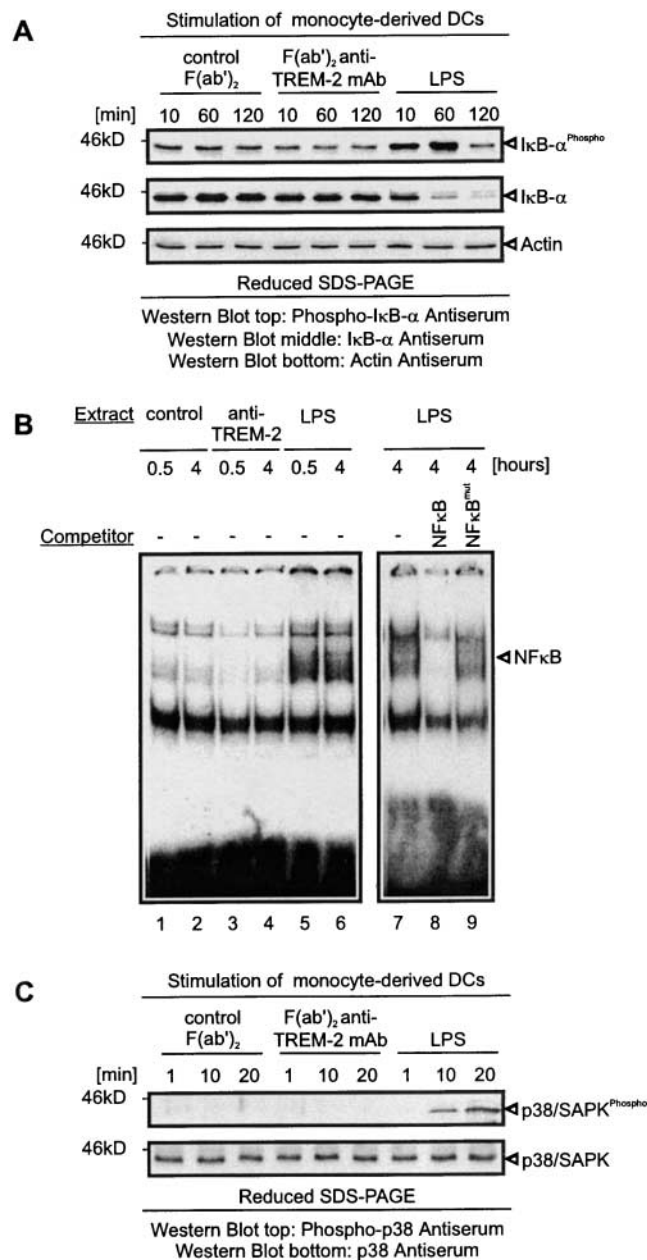
TREM-2 Does Not Activate IκBα/NF-κB or p38/SAPK Signaling Pathways. LPS-induced maturation of DCs requires the activity of NF-κB transcription factor (74–76). Prior to activation, NF-κB is retained in the cytoplasm through binding to the inhibitory IκB protein. Stimulation of cells with LPS leads to IκB kinase (IKK)-mediated phosphorylation of IκB. This is followed by rapid ubiquitination and proteolytic degradation of IκB, allowing nuclear translocation of NF-κB and binding to κB-promoter elements (30–33, 77). To study whether TREM-2 activates the IκBα/NF-κB pathway, we stimulated DCs through TREM-2 and analyzed the phosphorylation of IκBα by Western blot analysis. In addition, we assessed the nuclear translocation of NF-κB by searching for NF-κB-containing complexes in EMSAs. In striking contrast to LPS (72), antibody-mediated ligation of TREM-2 did not lead to phosphorylation and degradation of IκBα (Fig. 5 A) or nuclear translocation of NF-κB (Fig. 5 B). It was previously shown that LPS-induced maturation of DCs is also mediated by activation of p38/SAPK (72, 78). To see whether TREM-2 activates p38/SAPK, we crosslinked TREM-2 on DCs with F(ab')₂ 29E3 and analyzed tyrosine phosphorylation of p38/SAPK by Western blot analysis. In contrast to LPS, TREM-2 did not induce p38/SAPK tyrosine phosphorylation (Fig. 5 C).

Thus, the TREM-2-induced activation pathway is NF- κ B- and p38/SAPK independent.

TREM-2 Induces DC Maturation through an ERK- and PTK-dependent, NF- κ B, and p38/SAPK-independent Pathway. To further characterize the signaling molecules involved in TREM-2-mediated DCs maturation, DCs were incubated with inhibitors of ERK (PD98049), NF- κ B (TPCK), p38/SAPK (SB203580), and PTKs (PP2). Treated cells were stimulated with either F(ab')₂ anti-TREM-2 mAb, LPS, or immobilized IgG and subsequently analyzed for cell surface expression of maturation markers, such as CCR7, MHC class II, ICAM-1 CD83, CD40, and CD86. TREM-2 induced upregulation of CCR7, CD86, class II, and CD40, while cell surface expression of ICAM-1 and

CD83 was not increased (Fig. 6, red bars). Remarkably, TREM-2-induced expression of CCR7, CD86, class II, and CD40 was completely blocked by a PTK inhibitor and partially blocked by an ERK inhibitor. This differential inhibitory capacity suggests that PTK may activate downstream signaling molecules other than ERK, which concur to TREM-2-mediated DC maturation. Incubation of DCs with NF- κ B and p38/SAPK inhibitors had virtually no effect (Fig. 6, red bars).

LPS-induced maturation pathway was totally distinct from that mediated by TREM-2. LPS-induced upregulation of CCR7, MHC class II, ICAM-1 CD83, CD40, and CD86 (Fig. 6, green bars) (1). Incubation of DCs with NF- κ B and p38/SAPK inhibitors prevented LPS-induced maturation, whereas ERK inhibitor had a modest effect, as described previously (Fig. 6, green bars) (72, 78). Finally, engagement of FcR by immobilized IgG induced a maturation pattern, which was similar to that induced by TREM-2, with the exception of an upregulation of ICAM-1 (Fig. 6, blue bars). Incubation of DCs with PTK and ERK inhibitors resulted in total and partial inhibition of FcR-induced maturation, respectively. These observations provide evidence that TREM-2 mediates DC maturation by PTK/ERK-dependent pathways. These pathways overlap with those initiated by FcRs, but are distinct from the I κ B α /NF- κ B and p38/SAPK-dependent pathways triggered by LPS.



Discussion

We have shown that TREM-2 is an activating receptor expressed on monocyte-derived DCs, which activates PTK and ERK signaling through the association with DAP12, an ITAM-containing adaptor molecule (44, 45). TREM-2/DAP12-mediated signaling promotes survival of DCs

Figure 5. TREM-2 does not initiate I κ B α /NF- κ B- and p38/SAPK-dependent pathways. (A) Lack of I κ B α phosphorylation and degradation upon TREM-2 stimulation of monocyte-derived DCs. Monocyte-derived DCs were stimulated by LPS or plastic-bound F(ab')₂ anti-TREM-2 mAb or control F(ab')₂ for the indicated times. Protein lysates were tested for I κ B α phosphorylation and degradation by Western blot analysis. The same blot was sequentially stripped and reprobed with anti-phospho-I κ B α , anti-I κ B α , and anti-actin (loading control) antibodies. (B) Lack of NF- κ B translocation upon TREM-2 triggering. Monocyte-derived DCs were stimulated for the indicated time points as described in panel A and nuclear extracts were obtained. Radiolabeled NF- κ B consensus double-stranded oligonucleotides were incubated with the indicated nuclear extracts in the absence of competing oligonucleotides or in the presence of a 25-fold molar excess of wild-type (NF- κ B) or mutant (NF- κ B^{mut}) competing oligonucleotides. DNA-protein complexes were resolved by electrophoresis. The NF- κ B-containing complex is marked. (C) Absence of p38/SAPK phosphorylation upon TREM-2 stimulation of monocyte-derived DCs. Monocyte-derived DCs were stimulated for the indicated times as described in panel A. Protein lysates were tested for p38/SAPK phosphorylation (top panel) by Western blot analysis. The same blot was sequentially stripped and reprobed with anti-p38/SAPK antibodies (bottom panel). Arrows indicate proteins in all panels. Molecular weight markers are shown. To confirm proper stimulation of TREM-2/DAP12 in all experimental settings an aliquot of stimulated DCs was kept and tested after 48 h for upregulation of MHC class II and CD86 (data not shown).

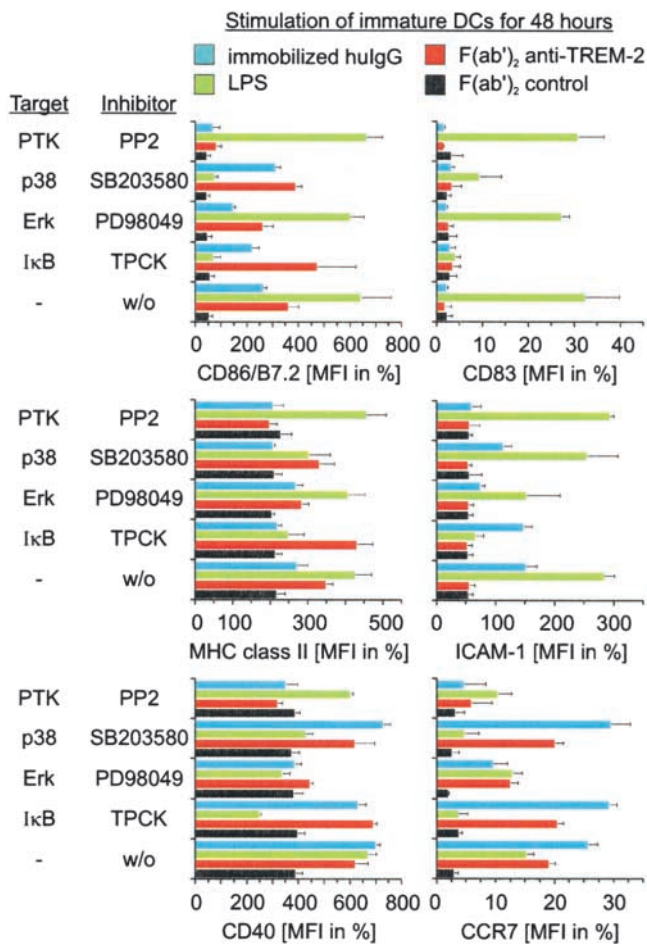


Figure 6. Comparison of TREM-2-, LPS/TLR-, and FcR-mediated maturation pathways. Monocyte-derived DCs were stimulated with plastic-bound control F(ab')₂ (black bars), F(ab')₂ anti-TREM-2 (red bars), human IgG (blue bars), or LPS (green bars) in the presence of inhibitors for Erk (PD98049), p38/SAPK (SB203580), PTK (PP2), IκBα degradation (TPCK), or an equal volume of DMSO as a control. After 48 h, cell surface expression of CD86 (top left panel), MHC class II (middle left panel), CD40 (bottom left panel), CD83 (top right panel), ICAM-1 (middle right panel), or CCR7 (bottom right panel) was determined by flow cytometry. Data shown are representative of four independent experiments and display the mean and standard deviation of three independent samples.

and upregulation of CCR7, MHC class II, CD86, and CD40. Compared with the classical DC activation triggered by LPS, the TREM2/DAP12 pathway does not lead to upregulation of ICAM-1 and CD83 or secretion of IL-12, and is entirely independent of NF-κB and p38/SAPK signaling. TREM2/DAP12-induced DC maturation is more similar to that initiated by the FcRs, through the association FcRγ, another ITAM-containing adaptor molecule (42). Indeed, here we have shown that FcR-mediated maturation is dependent on PTK and ERK signaling.

This study is the first to show that a DAP12-mediated pathway can activate human DCs. What could be the physiological significance of this maturation pathway? TREM-2 or other DAP12-associated receptors could synergize with cell surface receptors, which activate DCs

through NF-κB. This possibility is consistent with previous work on TREM-1, another DAP12-associated myeloid receptor (53), which is upregulated on neutrophils upon exposure to LPS and synergizes with LPS in promoting inflammatory responses to bacterial infections (79, 80). Thus, DAP12-associated DC receptors could amplify maturation signals transduced by other receptors, allowing for optimal antigen presentation. DAP12-mediated DCs activation could also be important in the normal homeostasis of DCs. TREM-2 induces upregulation of CCR7, which plays a pivotal role in directing DCs from the periphery to the T cell rich areas of draining lymph nodes (10–14). Thus, in the absence of pathogens, DAP12-associated receptors could regulate the homeostatic circulation of DCs from the periphery to the lymph nodes, allowing for renewal of lymph node DCs. In addition, TREM-2 induces upregulation of some T cell stimulatory molecules, such as MHC class II, CD40, and CD86. Thus, DAP12-mediated maturation of DCs may promote partial activation of T cells in the absence of exogenous antigens. This activation presumably is critical for the survival of T cells and the homeostasis of T cell populations (81).

We have demonstrated that crosslinking of TREM-2 promotes DC survival. This observation is consistent with previous demonstration that ERK signaling prevents apoptosis of LPS-stimulated DCs (71). In addition, we have evidence that TREM-1/DAP12 complex promotes survival of neutrophils and monocytes (unpublished data). Together, these observations suggest that DAP12-mediated pathways are critical for myeloid cell survival. Based on our data and previous studies in other systems, DAP12-mediated ERK activation is likely to induce phosphorylation of Bad or other Bcl-2 inhibitors (82–84). Once released from inhibition, Bcl-2 could translocate into the mitochondria and inhibit DC apoptosis (85–88).

The physiological functions of DAP12-mediated DC activation are consistent with the reported phenotypes of knock-in mice bearing a nonfunctional DAP12 and DAP12-deficient mice (57, 58). ITAM-deficient DAP12-knock-in mice showed an accumulation of DCs in mucocutaneous epithelia, associated with an impaired hapten-specific contact sensitivity (57). Our data suggest that this phenotype may be explained in part by a reduced ability of DCs to upregulate CCR7 expression and to respond to CCL19 and CCL21, affecting the migration of DCs to the T cell zone of draining lymph nodes. DAP12-deficient mice were resistant to EAE and resistance was associated with a strongly diminished production of IFN-γ by myelin-reactive CD4⁺ T cells (58). Our results suggest that this phenotype may be partly due to a reduced amplification of chemotactic and maturation signals by DAP12-associated receptors, resulting in an inadequate T cell priming in vivo.

Human TREM-2 is the first DAP12-associated receptor identified on DCs. TREM-2 is expressed on immature monocyte-derived DCs, but not on mature DCs or primary blood DCs. In addition, mouse TREM-2 transcripts were recently observed in cultured macrophage cell lines (54). Thus, more DAP12-associated receptors are likely to

be expressed on blood and tissues DCs. In addition, such receptors might be expressed not only in immature DCs, but also in mature DCs, regulating critical functions, such as IL-12 secretion. To fully understand the physiological functions of DAP12 in DCs, it will be important to identify all the DAP12-associated DC receptors, their level of expression in different maturation stages, and their ligands.

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