Non-T Cell Activation Linker (NTAL) Negatively Regulates TREM-1/DAP12-Induced Inflammatory Cytokine Production in Myeloid Cells[1]

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The engagement of triggering receptor expressed on myeloid cells 1 (TREM-1) on macrophages and neutrophils leads to TNF-α and IL-8 production and enhances inflammatory responses to microbial products. For signal transduction, TREM-1 couples to the ITAM-containing adapter DNAX activation protein of 12 kDa (DAP12). In general, ITAM-mediated signals lead to cell activation, although DAP12 was recently implicated in inhibitory signaling in mouse macrophages and dendritic cells. To date, signals downstream of the TREM-1 and DAP12 complex in myeloid cells are poorly defined. By analyzing receptor-induced tyrosine phosphorylation patterns, we discovered that the ligation of TREM-1 leads to tyrosine phosphorylation of the non-T cell activation linker (NTAL; also called linker of activation in B cells or LAB) in a myelomonocytic cell line and primary human granulocytes. Using RNA interference to decrease the expression levels of NTAL, we demonstrate that in NTAL knockdown cell lines the phosphorylation of ERK1/2 is enhanced. In addition, low levels of NTAL are correlated with decreased and delayed mobilization of Ca^{2+} after TREM-1 triggering. Most importantly, we demonstrate that NTAL acts as a negative regulator of TNF-α and IL-8 production after stimulation via TREM-1. Our results show that activation signals delivered via DAP12 can be counterbalanced by the adaptor NTAL, identifying NTAL as gatekeeper of TREM-1/DAP12-induced signaling in myeloid cells. ***The Journal of Immunology,*** **2007, 178: 1991–1999.**

Upon microbial infection, the innate immune system serves as the first line of defense by coordinating several cell types, including professional phagocytes (neutrophils and monocytes/macrophages) and NK cells (1). A delicate balance exerted by inhibitory and activating receptors is one key element of innate immune cell activation (2).

Many activating receptors have a short intracellular domain lacking intracellular signaling motifs and associate with adapter molecules, including CD3ζ and the FcγR chain or the DNAX activation protein of 12 kDa (DAP12)[3] (3, 4). These adapters contain an aspartic acid residue within their transmembrane regions to noncovalently pair with receptors harboring complementary, positively charged amino acid residues in their transmembrane domains. The cytoplasmic tail of these adapters contains one or more copies of an ITAM (peptide sequence Yxx(L/I)x_{6-8}Yxx(L/I), where x denotes any amino acid) (5, 6). The tyrosines within the ITAM motif are both necessary and sufficient for intracellular downstream signaling (7, 8).

The signaling adapter protein DAP12, also termed the killer cell-activating receptor (KAR)-associated protein (KARAP), was initially characterized as a disulfide-bonded homodimer in NK cells, coupling to the activating killer cell Ig-like receptor (KIR) KIR2D2 (9) and to p50 KAR (10). Meanwhile, the association of DAP12 with other activating receptors such as Ly49D, Ly49H (11–14), NKG2C, CD94 (15), the NKp44 receptor (16), the myeloid DAP12-associating lectin 1 (17), and the signal regulatory protein β1 (18, 19) has been demonstrated.

Screening for further DAP12-associated receptors led to the identification of a 30 kDa glycoprotein, triggering receptor expressed on myeloid cells 1 (TREM-1) (20). TREM-1 is expressed on the cell surface of blood neutrophils, subsets of monocytes, and tissue macrophages and is up-regulated upon bacterial and fungal infection (20). To date, ligands for TREM-1 are still unknown. However, Bouchon et al. (20) demonstrated that Ab ligation of TREM-1 on human granulocytes and monocytes leads to production of the proinflammatory cytokines TNF-α and IL-8. Furthermore, upon injection of a soluble mTREM-1-Ig-fusion protein in vivo, reduced inflammation and increased survival after induction of endotoxemia or septic shock was observed (21). Accordingly, DAP12 knockout mice display diminished susceptibility to septic shock, which further confirms that DAP12 amplifies inflammatory responses (22). In contrast, enhanced responses of DAP12-deficient macrophages to low concentrations of certain TLR ligands and enhanced clearance of bacterial infections in DAP12-deficient mice were reported (23), suggesting that DAP12 can also mediate inhibitory responses.

The engagement of TREM-1 transduces a signaling cascade initiated by the phosphorylation of tyrosines within the ITAM of

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[3] Abbreviations used in this paper: DAP12, DNAX activation protein of 12 kDa; DN, dominant negative; Grb2, growth factor receptor-bound protein 2; LAT, linker of activation in T cells; LM, laurylmaltoside; LNGFR, low affinity nerve growth factor receptor; NTAL, non-T cell activation linker; N4, NTAL-4; N5, NTAL-5; PLC-γ, phospholipase C-γ; pY, phosphotyrosine; SBP, streptavidin binding peptide; shRNA, short hairpin RNA; siRNA, small interfering RNA; S6H, streptavidin binding peptide-6×His; TREM-1, triggering receptor expressed on myeloid cells; U937-TD, U937 cells expressing TREM-1/DAP12; VC, empty vector control.

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DAP12 by Src family kinases. This enables the Src homology 2 domain-containing protein Syk to bind to DAP12 (9) and to subsequently transduce further downstream signals involving phospholipase Cγ (PLC-γ) and ERK1/2 (20). In addition, NK cell signaling via DAP12 after Ly49D ligation involves the ubiquitin ligase c-Cbl (24).

In general, ITAM-dependent signals are integrated and propagated by cytoplasmic and transmembrane adapter proteins. In T and NK cells, the linker of activation in T cells (LAT) was discovered as an essential transmembrane adapter, linking ZAP70/Syk to the activation of PLC-γ and ERK1/2 (25, 26). More recently, a structural and partially functional homologue of LAT, non-T cell activation linker (NTAL)/linker of activation in B cells (LAB), was described as being tyrosine phosphorylated upon cross-linking of the BCR, the Fcγ and Fcε receptors, and c-Kit (27–29). In resting T and B cells only LAT or NTAL, respectively, are expressed. However, NK cells, mast cells, and monocytes express both LAT and NTAL at distinct quantities and it is feasible that these adapters serve nonredundant functions. Indeed, it was shown that depending on the targets and NK activation status, the expression of both NTAL and LAT is required for high cytotoxicity and cytokine production of mouse NK cells (30). The relative contribution of these scaffolding adaptors in facilitating and fine tuning DAP12-mediated signaling in myeloid cells is still to be elucidated.

The focus of our study was to discover missing components in the TREM-1/DAP12 pathway in myeloid cells. By analyzing receptor-induced tyrosine phosphorylation patterns, we identified NTAL as being involved in TREM-1 signaling not only in the myelomonocytic cell line U937 but also in primary human granulocytes. Using small interfering RNA (siRNA)-mediated knockdown of NTAL, we demonstrate that NTAL is a negative regulator of TNF-α and IL-8 production after stimulation via TREM-1. Our results define NTAL as novel functionally important component in the signaling cascade downstream of TREM-1/DAP12 in myeloid cells and may help in the design of new strategies to combat infections and inflammatory diseases by modulating signals delivered by innate immune receptors.

Materials and Methods

Cells and cell culture

The myelomonocytic cell line U937 (American Type Culture Collection no. CRL-1593) and U937-derived cell lines were cultured in RPMI 1640 supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, and streptomycin (Invitrogen Life Technologies). The Phoenix Ampho (Phoenix-A) cell line (American Type Culture Collection no. SD 3443) was cultured in DMEM supplemented as described above for RPMI 1640. Primary granulocytes were isolated from human peripheral blood by using Polymorph Prep (PROGEN Biotechnik). The purity of isolated granulocytes amounted to >90% as determined by flow cytometry after staining with anti-CD15 and anti-CD11b monoclonal mAbs and by the distinct forward and side light scattering profile of granulocytes.

Vectors

The pMX-pie, pMX-neo, and pMXs-IG vectors were kindly provided by T. Kitamura (University of Tokyo, Tokyo, Japan) (31). pMXs-IN was generated by exchanging the GFP of pMXs-IG with a C-terminal truncated human low affinity nerve growth factor receptor (LNGFR) (32).

cDNA constructs

Human TREM-1 cDNA was amplified from cDNA prepared from primary human macrophages using the primers 5′-TATTATCGATGAACTCCG AGCTGCAACTAAATTAA-3′ (forward; containing a *Cla*I site) and 5′-ATAAGAATGCGGCCGCCTAGGGTACAAATGACCTCAGCG-3′ (reverse; containing a *Not*I site). This cDNA fragment was inserted into the retroviral vector pMX-neo containing a N-terminal CD8 leader sequence followed by a FLAG tag (pMX-neo-CD8L-FLAG-TREM-1).

Human DAP12 cDNA was cloned from human dendritic cell cDNA using the primers 5′-CGGGATCCATGGGGGGACTTGAACCCTGCA-3′ (forward; containing *Bam*HI) and 5′-GGAATTCTTTGTAATACGGCCT CTGTGTGTTG-3′ (reverse). This cDNA fragment was fused to a C-terminal FLAG tag contained in the retroviral vector pMX-puro.

pMXs-IN DAP12 Y64F,75F. DAP12 cDNA was amplified using primers containing an additional V5 tag and cloned into the retroviral vector pMXs-IN. The inactive DAP12 mutant contains two point mutations within the ITAM sequence (Y64F and Y75F) as described by Tomasello et al. (33) and was generated by site-directed mutagenesis using standard protocols.

NTAL-SBP6-×His fusion (NTAL-S6H). A C-terminal cassette containing a streptavidin binding peptide (SBP)-6×His tag was amplified via 5′-CCGCTCGAGATGGACGAAAAGACCACCGGC-3′ (containing *Xho*I) and 5′-TAAAGCGGCCGCTTAGTGATGGTGATGGTGATG-3′ (containing *Not*I) from plasmid m18, provided by U. Schwertassek (German Cancer Research Center, Heidelberg, Germany), and ligated into the vector pMXs-IN (pMXs-IN-S6H). cDNA of human NTAL was amplified from pEF-IRES-NTAL using the primers 5′-CCGGAATTTCATGAGCTCGG GGACTGAACTG-3′ (forward; containing *Eco*RI) and 5′-CCGCTCGA GGGCTTCTGTGGCTGCCAC-3′ (reverse; containing *Xho*I). cDNA encoding NTAL was fused to a SBP-6×His tag by insertion into pMXs-IN-S6H using *Eco*RI and *Xho*I restriction sites.

NTAL-Y136,193,233F-SBP-6×His fusion (NTAL Y3F-S6H). Tyrosine residues of NTAL at positions 136, 193, and 233 were exchanged for phenylalanines (Y3F-S6H) by site-directed mutagenesis following standard protocols using pMXs-IN-NTAL-S6H as a template.

Short hairpin RNA (shRNA) constructs

The RVH1 vector was provided by R. Medzhitov (Yale University, New Haven, CT) (34) and the human CD4 marker gene was replaced by a C-terminal truncated human LNGFR. The following oligonucleotides encoding shRNAs were used: GFP (35); NTAL-4 (N4; 5′-GATCCCCGTA CCAGAACTTCAGCAAATTCAAGAGATTTGCTGAAGTTCTGGTAC TTTTTGGAAC-3′); and NTAL-5 (N5; 5′-GATCCCCAGGACAAGCTG TTGCAATTTTCAAGAGAAATTGCAACAGCTTGTCCTTTTTTGGA AC-3′). These oligonucleotides were annealed and ligated into the RVH1 vector downstream of the H1 promoter. All oligonucleotides were synthesized by MWG Biotec.

Generation of stable cell lines — retroviral transduction

Stable U937-derived cells were obtained by retroviral infection. Thus, the plasmid of interest was transfected into Phoenix Ampho cells by using Lipofectamine 2000 (Invitrogen Life Technologies). Two days later the retrovirus-containing supernatant was mixed with Polybrene (Sigma-Aldrich) and used to transduce U937 cells by spin infection. Transduced cells were enriched by flow cytometry sorting using a FACSVantage (BD Biosciences) or by magnetic bead sorting using anti-LNGFR-conjugated microbeads (Miltenyi Biotec). All cell lines in this study were established as polyclonal cultures.

The cell line U937-TD (transduced with pMX-pie-DAP12 and pMX-neo-CD8L-FLAG-TREM-1 was generated and the following cell lines were all based on U937-TD cells (plus the construct indicated): U937-TD-DN DAP12 (pMXs-IN-DAP12 Y64,75F); U937-TD-VC-S6H (pMXs-IN-SBP-6×His); U937-TD-NTAL-S6H (pMXs-IN-NTAL-SBP-6×His); U937-TD-NTAL Y3F-S6H (pMXs-IN-NTAL Y3F-SBP-6×His); U937-TD-VC (empty RVH1); U937-TD-GFP (RVH1-GFP shRNA); U937-TD-N4 (RVH1-N4); and U937-TD-N5 (RVH1-N5). VC represents an empty vector control, and DN is dominant negative.

Antibodies

The following mAbs were used: allophycocyanin-conjugated anti-CD11c, PE-conjugated anti-CD86, anti-ERK-1, anti-growth factor receptor-bound protein 2 (Grb2), and PE-conjugated anti-LNGFR (all BD Biosciences); PE-conjugated goat $F(ab')_2$ anti-mouse IgG (BioSource International); anti-pERK1/2 (Thr^{202}/Tyr^{204}) (Cell Signaling Technology); anti-FLAG (M2) and anti-β-actin (both Sigma-Aldrich); $F(ab')_2$ of anti-FLAG (M2) (generated using the ImmunoPure IgG1 Fab and $F(ab')_2$ preparation kit); $F(ab')_2$ goat anti-mouse (Jackson ImmunoResearch); anti-phosphotyrosine (anti-pY) (clone 4G10; Upstate Biotechnology); anti-TREM-1 (R&D Systems); anti-NTAL (NAP-7) (27); and anti-HLA class I (clone W6/32) and anti-HLA class II (clone L243) (both provided by G. Moldenhauer, German Cancer Research Center, Heidelberg, Germany). Venimmun N (Aventis Behring) was used to block FcRs.

Stimulation of cells for signal transduction experiments

If not indicated otherwise, 2×10^7 cells in 250 μl of RPMI 1640 medium were stimulated with 20 μg/ml mAb at 37°C for 5 min. Subsequently, cells were placed on ice and 2 volumes of ice-cold PBS were added. For cross-linking experiments, 2×10^7 cells in (250 μl cold RPMI 1640 medium were first incubated on ice with the primary mAb (20 μg/ml) for 20 min, washed twice with 0.5 ml of prewarmed RPMI 1640 before the addition of $F(ab')_2$ goat anti-mouse as the secondary Ab, and the mixture was incubated at 37°C for 5 min. Primary granulocytes were incubated for 30 min on ice with 2.5 mg/ml Venimmun N before stimulation. After stimulation, cells were immediately pelleted at 16,000 $\times$ *g* for 30 s and washed with cold PBS. Cell pellets were lysed in a cold lysis solution containing 1% laurylmaltoside (LM lysis buffer) (27).

Immunoprecipitation, poststimulation, and reprobing

Phosphoproteins. Cells (4×10^7) were lysed in LM lysis buffer and tyrosine phosphorylated proteins were enriched by using 25 μl of anti-phosphotyrosine mAb (clone 4G10)-conjugated agarose (Upstate Biotechnology). Bound proteins were eluted with 2× concentrated Laemmli buffer and analyzed by SDS-PAGE followed by immunoblotting. For the reprobing of immunoblots, membranes were treated with a stripping solution (100 mM 2-ME, 2% SDS, and 62.5 mM Tris) for 25 min at 65°C and subsequently washed several times with TBST and blocked in 5% BSA in TBST overnight.

NTAL was immunoprecipitated from postnuclear lysates of 4×10^7 cells solubilized in cold LM lysis buffer. For immunoprecipitation, 25 μl of cyanogen bromide-activated Sepharose 4B beads (GE Healthcare) coupled to NAP-7 mAb (following the manufacturer's instructions) were added per sample and rotated overnight at 4°C. After washing in lysis buffer, bound proteins were eluted with 2× concentrated Laemmli buffer and analyzed by SDS-PAGE followed by immunoblotting.

NTAL-S6H fusion proteins. Cells (U937-TD-NTAL-S6H or U937-TD-NTAL Y3F-S6H, 1×10^8 cells/sample) stably expressing a NTAL fusion protein were lysed with LM lysis buffer. NTAL-S6H fusion proteins and associated proteins were immunoprecipitated by using 150 μl of streptavidin-conjugated Sepharose (GE Healthcare), and lysates and beads were rotated for 2 h at 4°C. After washing with lysis buffer, bound proteins were eluted with 4 mM biotin (Sigma-Aldrich) in TBS for 15 min at 4°C. Laemmli buffer (10×) without 2-ME was added, and samples were analyzed by SDS-PAGE followed by immunoblotting.

ERK phosphorylation

ERK phosphorylation was determined after stimulation of 1×10^6 cells with anti-FLAG or anti-HLA class I (control) mAb for the indicated times. After three freeze and thaw cycles, cells were lysed with LM lysis buffer and resolved by SDS-PAGE, followed by immunoblotting using anti-pERK1/2 or anti-ERK1 mAb. Protein visualization was performed with the chemiluminescence mode of a LumiImager (Roche Diagnostics). Quantification of the protein signals was performed by using the LumiAnalyst software from Roche Diagnostics and ratios between pERK1/2 and ERK1 were calculated.

Ca^{2+} flux

Cells (5×10^6/ml) were loaded with 4 μM Indo-1 acetoxymethyl ester (Molecular Probes) in RPMI 1640 medium without FCS. Cells were washed twice for 5 min at 200 $\times$ *g* with RPMI 1640 medium without FCS and kept at 37°C for 30 min. Ca^{2+} flux was induced with anti-FLAG (20 μg/ml,), anti-TREM-1 (20 μg/ml), anti-HLA class I mAbs (20 μg/ml), or ionomycin (1 μg/ml) (Sigma-Aldrich) and measured by using a FACSVantage SE (BD Biosciences) flow cytometer.

Cell stimulation and cytokine measurement

Cells were stimulated for the indicated times on plates precoated with anti-FLAG (10 μg/ml) or a combination of anti-FLAG and LPS (from *Escherichia coli* serotype 0111:B4; 10 μg/ml) (Sigma-Aldrich). Triplicates of supernatants were harvested at the indicated time points and TNF-α and IL-8 were measured by specific ELISAs (R&D Systems) following the instructions of the manufacturer.

Results

Generation and characterization of a U937 cell line ectopically expressing TREM-1 and DAP12 (U937-TD)

To study signaling events downstream of DAP12 in myeloid cells, we established a myelomonocytic cell line (U937) that ectopically expresses TREM-1 and DAP12, termed U937-TD. U937 cells do not express endogenous TREM-1 and have very low amounts of endogenous DAP12 as determined by RT-PCR and/or flow cytometry (data not shown). First, a retroviral construct encoding DAP12 containing internal ribosome entry site (IRES)-GFP was transduced into U937 cells. After the selection of cells expressing high levels of DAP12 as determined by GFP expression, cells were transduced with a plasmid containing N-terminal FLAG-tagged TREM-1. After transduction of both DAP12- and TREM-1- containing plasmids, high levels of FLAG-tagged TREM-1 were detected on the cell surface by flow cytometry (Fig. 1*A*, *left panel*). Hardly any TREM-1 was observed on the cell surface in the absence of ectopic expression of DAP12 (data not shown), confirming that DAP12 is required in abundance for optimal cell surface expression of TREM-1. U937-TD cells stained at high levels with mAbs directed against HLA class I or HLA class II, which were used as controls in subsequent experiments (Fig. 1*A*, *right panel*).

We further established that stimulation via TREM-1/DAP12 leads to the activation of U937-TD cells. The triggering of TREM-1 by plate-bound anti-FLAG mAb induced cellular differentiation, as determined by up-regulation of the differentiation and activation markers CD11c and CD86 (Fig. 1*B*). Up-regulation of differentiation markers was further enhanced by the engagement of TREM-1 in combination with LPS. Incubation with LPS by itself, however, did not lead to up-regulation of CD11c or CD86 (data not shown). Plate-bound anti-HLA class I or anti-HLA class II mAbs did not induce cell differentiation, indicating that the effects seen were due to the TREM-1/DAP12 pathway. Furthermore, U937-TD cells produced low levels of TNF-α and IL-8 after triggering via plate-bound anti-FLAG mAb. No detectable levels of TNF-α and IL-8 were measured after stimulation with LPS alone for 4, 8, or 16h (data not shown). However, highly increased levels of TNF-α and IL-8 were detected when TREM-1 was engaged in combination with LPS (Fig. 1*C*).

TREM-1 stimulation leads to tyrosine phosphorylation of NTAL

Activation of TREM-1 in human monocytes stimulates tyrosine phosphorylation of various proteins including DAP12 (12 kDa), ERK1/2 (~40 kDa), and PLC-γ (~100 kDa) (20). We aimed to identify additional proteins that become tyrosine-phosphorylated after the triggering of TREM-1/DAP12 in myeloid cells. To this end, U937-TD cells were placed in medium alone or in medium containing an anti-FLAG mAb or an isotype-matched anti-HLA class II mAb. Then, tyrosine phosphorylated proteins were enriched from postnuclear lysates by immunoprecipitation and resolved by SDS-PAGE. Immunoblotting with an anti-pY mAb revealed that several proteins became tyrosine- phosphorylated upon TREM-1 stimulation, but not after stimulation with the isotype-matched anti-HLA class II mAb (Fig. 2*A*, *upper panel*). Our further investigation focused on a phosphorylated protein with an apparent molecular mass of 23 kDa (pYp23 kDa). Because the 26.5-kDa protein NTAL was previously identified to be tyrosine phosphorylated upon cross-linking of the BCR, FcγRI, or FcεRI, all of which are coupled to ITAM-containing adapters (27, 28, 36, 37), we asked whether the pYp23 kDa could be NTAL. Indeed, immunoblotting using a mAb specific for NTAL confirmed that the pYp23 kDa band corresponded to NTAL (Fig. 2*A*, *bottom panel*). Because anti-HLA class I and anti-HLA class II mAbs bind at high levels to the U937-TD cells (Fig. 1*B*) and their Fc regions bind to FcRs expressed on U937-TD cells, stimulation with anti-HLA class I or HLA class II mAbs was used as control (see Figs. 2–4 and 6). No pYp23 kDa was detectable after stimulation with anti-HLA class II or anti-HLA class I mAbs (Fig. 2*A* and data not shown). To further exclude that the observed signal resulted from

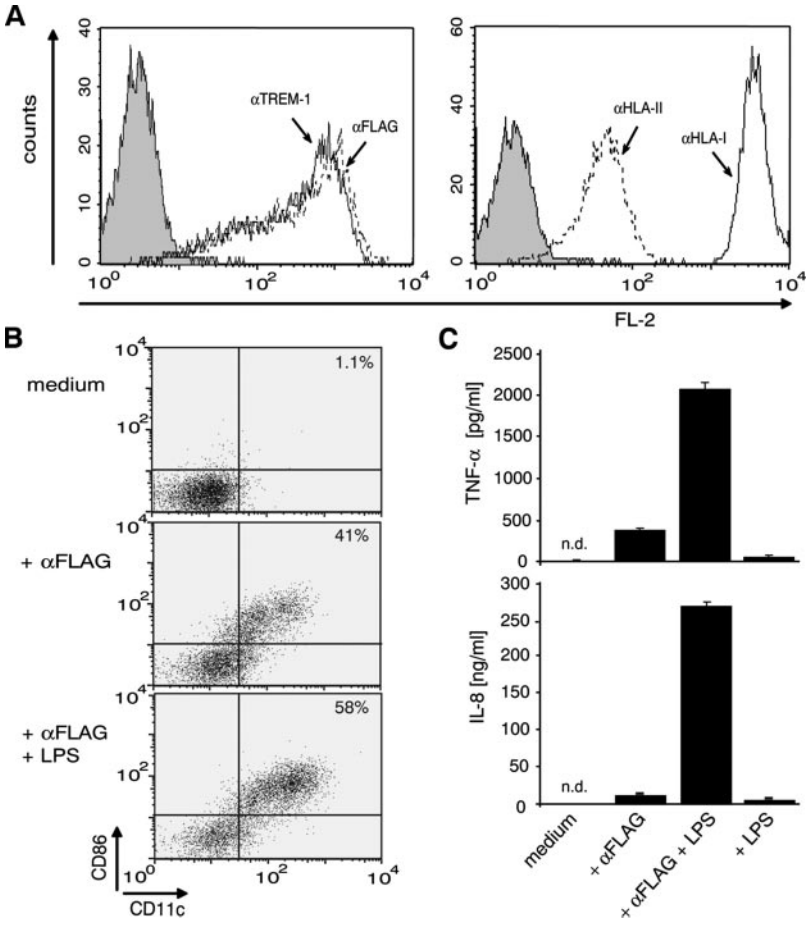

FIGURE 1. Generation and characterization of U937-TD, a U937 cell line ectopically expressing TREM-1 and DAP12. *A*, U937-TD cells, stably transduced with plasmids containing N-terminally FLAG-tagged TREM-1 and DAP12 were stained with an anti-TREM-1 mAb (solid line, *left panel*) or an anti-FLAG mAb reactive with FLAG-tagged TREM-1 (broken line, *left panel*) and analyzed by flow cytometry. In the *right panel*, U937-TD cells were stained with anti-HLA class I (solid line) or anti-HLA class II (broken line) mAbs, respectively. Staining with the isotype matched control mAb is depicted as shaded histograms. *B*, U937-TD cells were left in medium (*upper panel*), stimulated with plate-bound anti-FLAG mAb alone (*middle panel*) or plate-bound anti-FLAG (αFLAG) mAb in combination with LPS (*lower panel*) for 48 h. Dot plots show expression of CD86 and CD11c after stimulation with the indicated stimuli. *C*, U937-TD cells were incubated with plate-bound anti-FLAG mAb and LPS (αFLAG + LPS), each stimulus alone, or placed in medium only. Supernatants were harvested after 48 h and analyzed by ELISA for TNF-α (*top panel*) or IL-8 (*bottom panel*). n.d., Not detectable. One representative experiment of three experiments is shown in the figure.

FcR signaling, U937-TD cells were stimulated with $F(ab')_2$ of the anti-FLAG mAb, alone or cross-linked with goat anti-mouse $F(ab')_2$. $F(ab')_2$ fragments of the anti-FLAG mAb alone were not sufficient to induce phosphorylation of NTAL, whereas their cross-linking led to NTAL phosphorylation (Fig. 2*B*). These data indicate that the signal observed in our experiments after the ligation of TREM-1 was specific for the TREM-1/DAP12 pathway and, apparently, not secondary to signaling via FcRs.

We examined whether triggering with a mAb specific for TREM-1 yielded similar results as obtained by triggering with an anti-FLAG mAb. Fig. 2*B* illustrates that NTAL phosphorylation was detected not only after triggering with the anti-FLAG but also after triggering with the anti-TREM-1 mAb. To demonstrate that the observed phosphorylation of NTAL was not restricted to the cell line used in our study, we examined NTAL phosphorylation in primary human granulocytes isolated from the peripheral blood of healthy donors. Indeed, the stimulation of primary granulocytes with anti-TREM-1, but not with anti-HLA class I mAbs, led to tyrosine phosphorylation of NTAL (Fig. 2*C*), confirming our previous results in primary cells. In addition, NTAL phosphorylation after TREM-1 triggering was observed in PBMCs, suggesting that NTAL phosphorylation occurs not only in primary human granulocytes but also in primary human monocytes (data not shown).

TREM-1 lacks a signaling motif in its cytoplasmic domain and couples with the adapter DAP12 to mediate downstream signals. To determine whether NTAL only becomes phosphorylated after activation of the TREM-1/DAP12 complex, we generated the cell line U937-TD-DN DAP12, overexpressing an inactive form of DAP12 that was previously described as a loss-of-function mutant by Tomasello et al. (33). U937-TD-DN DAP12 cells expressed similar levels of FLAG and TREM-1 on the cell surface as compared with U937-TD cells (data not shown). After the engagement of TREM-1 via anti-FLAG mAb, no NTAL phosphorylation was detected in U937-TD-DN DAP12 cells (Fig. 2*D*). Taken together, our data demonstrate that NTAL phosphorylation requires functional DAP12.

Stimulation-dependent association of Grb2 with NTAL

Previous studies revealed that NTAL associates with Grb2 in B and monocytic cell lines (27), as well as bone marrow-derived mast cells (36, 37). This interaction was dependent on three membrane-distal tyrosines (Y136, Y193, and Y233) of NTAL, and these residues were implicated in augmenting signals induced by the BCR to generate Ca^{2+} mobilization (38, 39). Therefore, we asked whether NTAL also associates with Grb2 after TREM-1 stimulation. For these studies, we established cell lines stably expressing a NTAL-streptavidin binding peptide-6×His fusion protein (NTAL-S6H) composed of the wild-type protein or the Y3F mutant protein in which the Y136, Y193, and Y233 were replaced by phenylalanine. All cell lines used displayed similar amounts of FLAG and TREM-1 on the cell surface, as well as similar amounts of NTAL fusion protein expression (data not shown). Fig. 2*E* illustrates that upon TREM-1 ligation, NTAL phosphorylation is observed in the wild type but not in the Y3F mutant protein, suggesting that phosphorylation is mainly restricted to the tyrosine residues at positions 136, 193, and 233. Furthermore, the association of Grb2 was only seen when NTAL was phosphorylated (Fig. 2*E*). As control, a cell line expressing the empty vector containing the S6H tag (U937-TD-VC-S6H) was stimulated and neither phosphorylated NTAL nor Grb2 association was detected. Reprobing

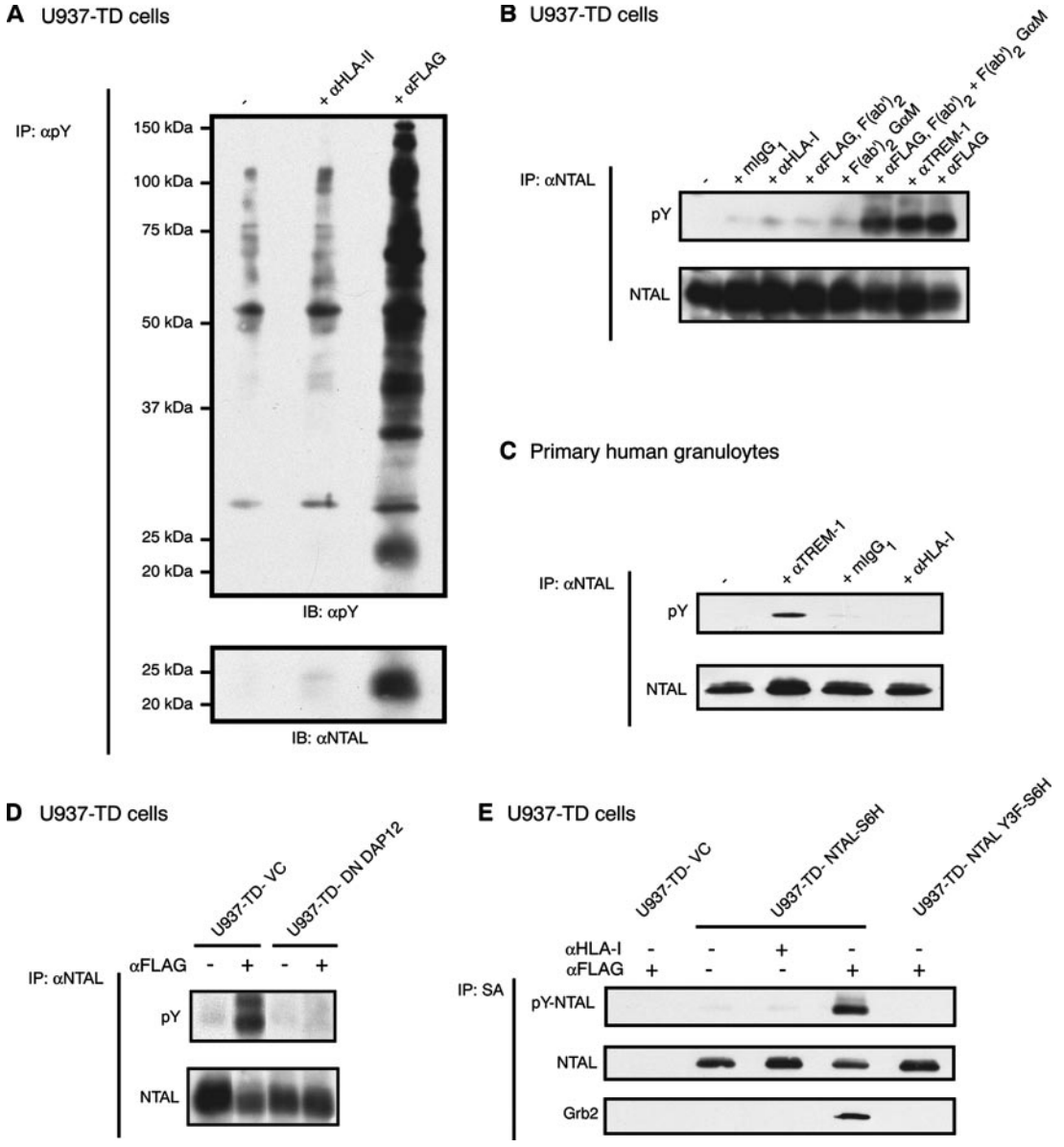

FIGURE 2. Identification and validation of NTAL as a component in the TREM-1/DAP12 pathway. *A*, U937-TD cells were left untreated, incubated with isotype-matched anti-HLA class II mAb, or stimulated with anti-FLAG (αFLAG) mAb for 5 min. Postnuclear lysates were immunoprecipitated (IP) with 4G10 (anti-pY)-coupled beads and tyrosine-phosphorylated proteins were visualized by blotting with an anti-pY mAb (*upper panel*). Phosphorylated NTAL was identified by re-probing the blot with an anti-NTAL mAb (*lower panel*). *B–E*, After stimulation for 5 min with the indicated stimuli, U937-TD cells (*B*), primary human granulocytes (*C*), U937-TD and U937-TD-DN DAP12 cells (*D*), or U937-TD-VC-S6H, U937-TD-NTAL-S6H, and U937-TD-NTAL Y3F-S6H cells (*E*) were lysed and postnuclear lysates were immunoprecipitated with covalently coupled anti-NTAL beads (*B–D*) or streptavidin (SA) beads (*E*). *B–E*, Phosphorylation was detected by blotting with anti-pY mAb (*upper panels*). The amount of NTAL precipitated was determined by reprobing the blots with an anti-NTAL mAb (*B–D*, *bottom panels*; *E*, *middle panels*). *E*, Reprobing was performed using an anti-Grb2 mAb (*bottom panel*). One representative experiment of five experiments is shown for *A*, *B*, *D*, and *E*, and one of two experiments for *C* is shown.

the blot with anti-NTAL mAb showed that similar levels of the NTAL fusion protein were immunoprecipitated (Fig. 2*E*, *middle panel*). These results demonstrate a stimulation-dependent NTAL-Grb2 association downstream of DAP12.

Generation and characterization of NTAL knockdown U937-TD cell lines

We observed that NTAL was phosphorylated after the engagement of TREM-1/DAP12. To further investigate the functional role of NTAL in the TREM-1/DAP12 pathway, we established the cell lines U937-TD-N4 and U937-TD-N5 in which NTAL expression was decreased by RNA interference. To this end, we transduced U937-TD cells with two different constructs (termed N4 and N5) containing oligonucleotides that encode shRNAs directed against NTAL. Vectors containing oligonucleotides based on the sequence of GFP or the empty vector were used as controls. All generated cell lines expressed FLAG-tagged TREM-1 at similar levels on the cell surface (data not shown). Fig. 3*A* illustrates that GFP siRNA or the vector alone did not affect expression of NTAL, as determined by immunoblotting using a specific anti-NTAL mAb. In contrast, transduction of the constructs delivering shRNA directed against NTAL significantly decreased levels of NTAL expression. After balancing the expression levels of NTAL against the housekeeping protein β-actin using a LumiImager, we confirmed highly reduced levels of NTAL (reduction by ~94% compared with vector control in both U937-TD-N4 and -N5 cell lines) (data not

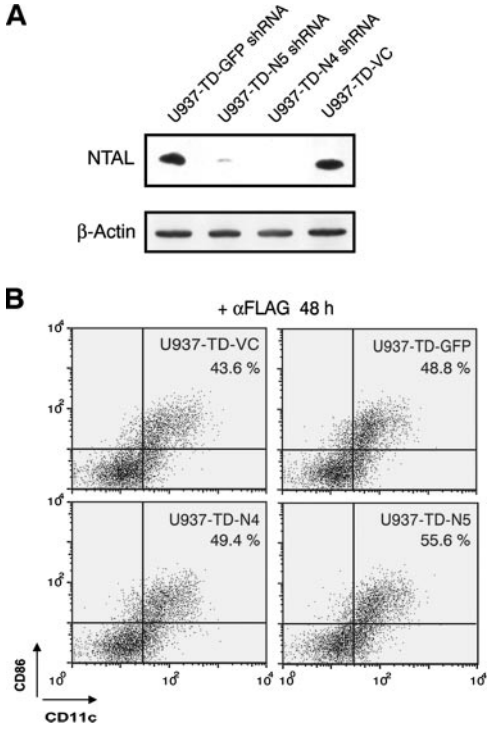

FIGURE 3. Generation of U937-TD NTAL knockdown cell lines. U937-TD cells, stably transduced with an empty vector (U937-TD-VC) or vectors delivering two distinct shRNAs directed against NTAL (U937-TD-N4 or -N5) or against GFP (U937-GFP), were established as described in *Materials and Methods*. *A*, Cell lysates of U937-TD-derived cells were resolved on SDS-PAGE and blotted with anti-NTAL (*upper panel*) or anti-β-actin mAbs (*lower panel*), respectively. *B*, U937-TD-derived cells were stimulated with plate-bound anti-FLAG (αFLAG) mAb for 48 h, and the expression of CD11c and CD86 after stimulation with anti-FLAG was determined by flow cytometry. One representative experiment of six independently performed experiments is shown.

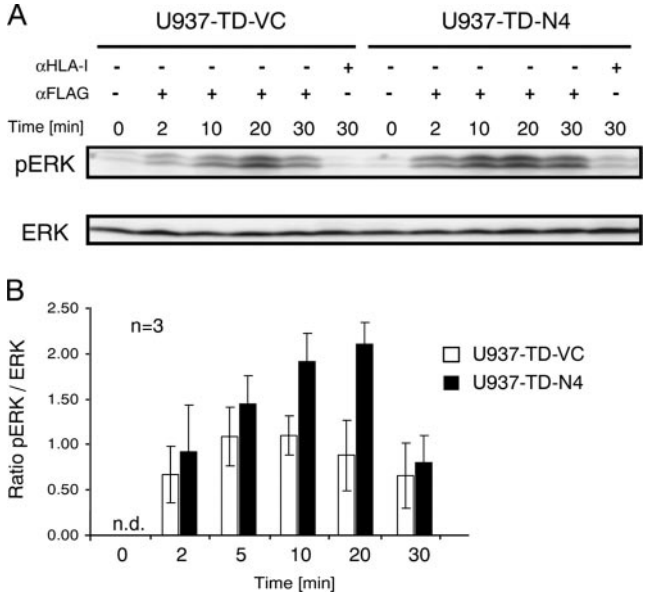

FIGURE 4. ERK1/2 phosphorylation after TREM-1 stimulation. *A*, Lysates of U937-TD-VC and U937-TD-N4 cell lines, triggered with anti-FLAG (αFLAG) mAb for the indicated times or incubated with the isotype-matched anti-HLA (αHLA) class I mAb for 30 min, were analyzed by immunoblotting with anti-phosphorylated ERK1/2 (pERK; *upper panel*) or anti-ERK 1 (*lower panel*) mAbs. *B*, LumiImager signals of pERK1/2 and ERK 1, obtained from three independent experiments ($n = 3$), were quantified using the LumiAnalyst software and the ratio between pERK1/2 and ERK 1 was determined.

shown). Despite reduced NTAL levels, the cell lines differentiated to similar extents upon stimulation via TREM-1 as determined by up-regulation of CD11c and CD86 expression (Fig. 3*B*).

Functional role of NTAL in downstream signaling of TREM-1/DAP12

TREM-1 ligation leads to phosphorylation of ERK1/2 and Ca^{2+} mobilization in primary human monocytes (20). Thus, we investigated whether NTAL affects phosphorylation of ERK1/2 by stimulating U937-TD-VC and U937-TD-N4 cells with anti-FLAG or anti-HLA class I mAbs for the indicated time periods (Fig. 4). Fig. 4*A* shows a slight but consistent enhancement of ERK1/2 phosphorylation in U937-TD-N4 cells as compared with U937-TD-VC cells. The increase in pERK1/2 levels in the U937-TD-N4 cell line was most pronounced at 10 and 20 min after stimulation. Stimulation with anti-HLA class I for 30 min did not induce ERK phosphorylation (Fig. 4*A*). Enhanced levels of pERK1/2 in U937-TD-N4 cells were also confirmed by the quantification of data obtained in three independently performed experiments using a LumiImager (Fig. 4*B*). These data indicate that at certain time points NTAL negatively modulates ERK1/2 phosphorylation.

Next, we determined whether TREM-1-induced Ca^{2+} flux was altered in the NTAL knockdown cell lines. After triggering with anti-FLAG mAb, Ca^{2+} flux was significantly reduced but prolonged in U937-TD-N4 and -N5 cells in comparison to U937-TD or U937-TD-VC cells (Fig. 5). The ligation of TREM-1 with the anti-TREM-1 mAb showed similar results (data not shown). Stimulation of these cell lines with ionomycin resulted in comparable levels of Ca^{2+} mobilization, indicating that all cell lines were able to mobilize Ca^{2+} to similar extents (data not shown). The observed Ca^{2+} mobilization was specific for stimulation via TREM-1, because stimulation with anti-HLA class I or the stimulation of parental U937 cells did not lead to a detectable Ca^{2+} flux. In addition, no difference in Ca^{2+} flux was observed after stimulation of U937-TD-GFP and U937-TD-VC cells (Fig. 5). In summary, in cell lines with decreased levels of NTAL, a reduced but prolonged Ca^{2+} flux was observed after TREM-1 triggering.

NTAL negatively regulates the production of TNF-α and IL-8

The engagement of TREM-1 triggers the secretion of TNF-α and IL-8 in human monocytes (20). To examine whether NTAL has an effect on the production of these cytokines, U937-TD-VC, U937-TD-N4, and U937-TD-N5 cells were incubated in the presence or absence of plate-bound anti-FLAG. After triggering of TREM-1

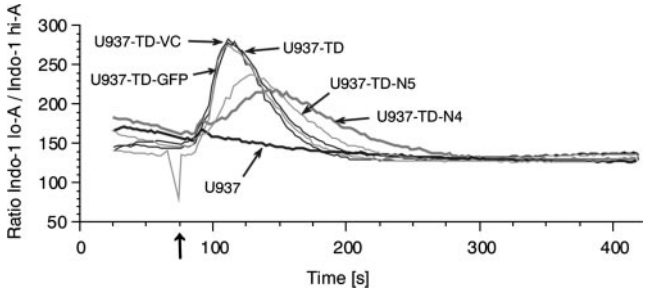

FIGURE 5. Reduced Ca^{2+} flux in U937-TD-N4 and U937-TD-N5 cells. Ca^{2+} mobilization of Indo-1 acetoxymethyl ester-labeled U937-TD derived cell lines was analyzed by flow cytometry. Cells were stimulated with anti-FLAG mAb at the time point indicated by the arrow. One representative experiment of four experiments is shown.

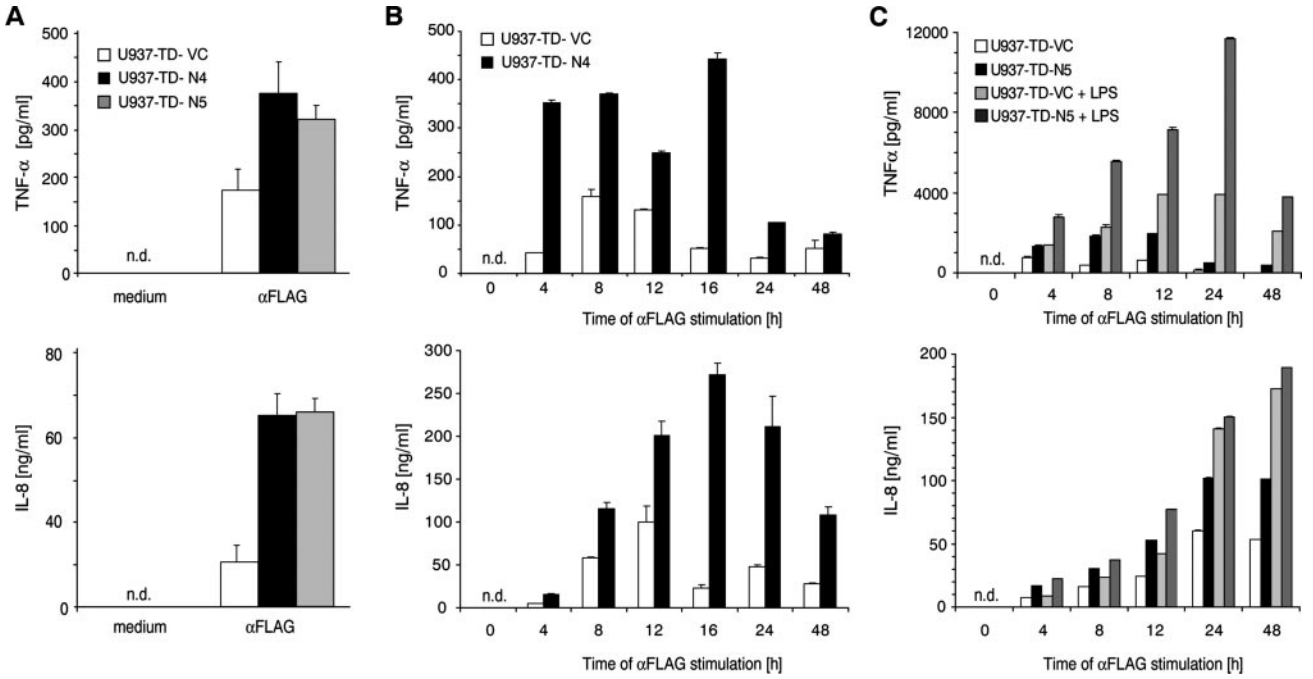

FIGURE 6. Enhanced TNF-α and IL-8 production in U937-TD-N4 and U937-TD-N5 after TREM-1 triggering. *A*, U937-TD-VC, U937-TD-N4, or U937-TD-N5 cells were incubated for 24 h with either plate-bound anti-FLAG (αFLAG) mAb or medium only. *B*, U937-TD-VC or U937-TD-N4 cells were incubated with plate-bound anti-FLAG mAb for indicated time periods. *C*, U937-TD-VC or U937-TD-N5 cells were incubated with plate-bound anti-FLAG mAb only or in combination with LPS for the indicated time periods. Supernatants were analyzed by ELISA for TNF-α (*top panels*) or IL-8 (*bottom panels*). One representative experiment of four (*A* and *B*) or two (*C*) experiments is shown. n.d., Not detectable.

for 24 h, the release of TNF-α, as well as IL-8, was strongly enhanced in U937-TD-N4 and U937-TD-N5 cells (Fig. 6*A*) as compared with the vector control cell line. U937-TD-N4 or U937-TD-N5 cells released significantly increased levels of TNF-α and IL-8 upon the stimulation of TREM-1 for different time periods (Fig. 6, *B* and *C*). Upon stimulation via TREM-1 in combination with LPS, U937-TD-N5 released elevated amounts of TNF-α and IL-8 as compared with U937-TD-VC at every time point tested (Fig. 6*C*). Neither TNF-α nor IL-8 was detected after stimulation with LPS or anti-HLA class I mAb at any time point (data not shown). Together, our results identify NTAL as a negative regulator of TNF-α and IL-8 production.

Discussion

Signaling events downstream of the ITAM-containing adapter DAP12 and their functional impact on myeloid cell activation are incompletely understood. In this study, we discovered that the transmembrane adapter protein NTAL is a novel component of the TREM-1/DAP12 pathway. Our study demonstrates that in myeloid cells NTAL functions as a molecular gatekeeper conducting inflammatory cytokine production in response to triggering via TREM-1/DAP12.

NTAL was first identified as a structural and partially functional homologue of LAT expressed in monocytes and NK, mast, and B cells (27). Similar to LAT, NTAL consists of a short extracellular domain, a single hydrophobic transmembrane domain, and a cytoplasmic domain with nine tyrosines being conserved from mice to humans. To date, phosphorylation of NTAL was reported to occur after the triggering of the ITAM-coupled receptors BCR, FcγR, and FcεR (27, 28, 36, 37) and also after c-Kit aggregation in mast cells (29). Our studies show that triggering of another ITAM- mediated pathway, TREM-1/DAP12, also leads to the phosphorylation of NTAL. We demonstrate that the engagement of TREM-1 not only induces NTAL phosphorylation in the transduced cell line U937-TD but also in primary human granulocytes.

Because NTAL was shown to modulate ITAM-mediated signaling and function in B cells, NK cells, and mast cells, we analyzed the involvement of NTAL in signaling events downstream of TREM-1/DAP12 in myeloid cells. Thus, we established myelomonocytic cell lines with diminished NTAL expression levels by siRNA-mediated knockdown. In U937 cells with reduced NTAL expression levels, we observed that the phosphorylation of ERK1/2 was delayed but enhanced after 10–20 min of stimulation. In contrast, in the mouse B cell line A20 with decreased NTAL expression, reduced ERK phosphorylation was observed 5 min after BCR triggering (28). In these experiments, however, the impact of NTAL on ERK1/2 phosphorylation at later time points, which in our study show enhanced levels of phosphorylated ERK1/2, was not investigated.

Furthermore, data obtained in cell lines with reduced NTAL expression demonstrated decreased but prolonged Ca^{2+} mobilization after stimulation via TREM-1. Our results are in accordance with siRNA-mediated NTAL knockdown studies in A20 cells (28). Furthermore, NTAL$^{-/-}$ DT40 chicken B cells exhibited less intracellular and less extracellular Ca^{2+} flux (39). Rescue studies in these NTAL$^{-/-}$ cells using a NTAL mutant incapable of binding Grb2 demonstrated that the NTAL-Grb2 interaction is required to relocalize Grb2 to lipid rafts, thus overcoming the inhibitory role of Grb2 in Ca^{2+} mobilization (39). In our studies we observed a stimulation-dependent association of NTAL with Grb2. These data suggest that NTAL also positively regulates Ca^{2+} flux in myeloid cells by abrogating the inhibitory effect of Grb2. In contrast, Wang et al. (40) observed a slight increase in Ca^{2+} mobilization and proliferation upon BCR cross-linking using primary NTAL$^{-/-}$ B cells. Moreover, in mast cells of NTAL$^{-/-}$ mice, increased levels of intracellular Ca^{2+} were detected after FcεRI triggering (36, 37). Interestingly, in contrast to LAT, NTAL does not bind to PLC-γ (41), which plays an important role for Ca^{2+} mobilization (42).

Therefore, we suggest that the distinct effects of NTAL on Ca^{2+} flux may depend on the presence of additional modulator proteins in the different cell types evaluated.

Most importantly, we demonstrate that NTAL negatively regulates the production of TNF-α and IL-8 after TREM-1 stimulation. Cellular differentiation triggered via TREM-1/DAP12 was not affected by decreased levels of NTAL, indicating that NTAL might differentially control cytokine production and cell differentiation. It has been shown recently that after stimulation with LPS elevated levels of ERK phosphorylation correlated with the hyperproduction of TNF-α (43). We also assume in our study that the increased production of TNF-α by the NTAL knockdown cell lines could be secondary to the elevated levels of ERK phosphorylation observed in these cell lines. The question of whether there is a direct link between the observed reduced Ca^{2+} flux and the increase in TNF-α and IL-8 production in cells with decreased levels of NTAL needs to be investigated further. Recent studies suggested that LAT and its structural homologue NTAL can exert overlapping and distinct functions. In $LAT^{-/-}$ mice, the reconstitution of NTAL leads to a partial rescue of T cell development (28) and, to a certain extent, compensates for defects in TCR signaling (27). However, mice deficient both in LAT and NTAL show much more severe phenotypes with regard to NK cell-mediated cytotoxicity (30) and mast cell degranulation (36) as compared with mice in which either LAT or NTAL was individually knocked out. Resting T and B cells express LAT and NTAL, respectively. In mast cells, NK cells, and macrophages, both LAT and NTAL are expressed at distinct levels. U937 cells express NTAL, but only very low levels of LAT were detectable (44), 80% less in our cell lines than in Jurkat T cells (data not shown). Therefore, we assume that in our experiments low levels of LAT were not able to replace the function of NTAL.

Previously, Bouchon and colleagues reported that injection of a soluble TREM-1 fusion protein ameliorated LPS-induced septic shock (21). In addition, the engagement of TREM-1 on primary granulocytes leads to the production of TNF-α and IL-8 (20), indicating that TREM-1 may play a crucial role in amplifying inflammatory diseases and septic shock. Our results demonstrate that the transmembrane adapter NTAL has the potential to negatively regulate inflammatory cytokine production after the triggering of TREM-1/DAP12 in myeloid cells, which might contribute to new strategies for treating inflammatory diseases and septic shock.

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Disclosures

The authors have no financial conflict of interest.

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