A Disintegrin and Metalloprotease (ADAM) 10 and ADAM17 Are Major Sheddases of T Cell Immunoglobulin and Mucin Domain 3 (Tim-3) *

Katja Möller-Hackbarth 1,2, Christin Dewitz 3, Olga Schweigert 1, Ahmad Trad 1, Christoph Garbers 3, Stefan Rose-John 1, and Jürgen Scheller 4,5

From the 4 Institute of Biochemistry, Medical Faculty, Christian-Albrechts-University, 24098 Kiel, Germany and the 5 Institute of Biochemistry and Molecular Biology II, Medical Faculty, Heinrich-Heine-University, 40225 Düsseldorf, Germany

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Background: Membrane-bound Tim-3 regulates immune responses.
Results: Soluble Tim-3 is generated by A disintegrin and metalloprotease (ADAM) 10 and ADAM17.
Conclusion: ADAM proteases are involved in Tim-3 processing.
Significance: Shedding of Tim-3 influences immune cell activation.

T cell immunoglobulin and mucin domain 3 (Tim-3) dampens the response of CD4+ and CD8+ effector T cells via induction of cell death and/or T cell exhaustion and enhances the ability of macrophages to clear pathogens via binding to galectin 9. Here we provide evidence that human Tim-3 is a target of A disintegrin and metalloprotease (ADAM)-mediated ectodomain shedding resulting in a soluble form of Tim-3. We identified ADAM10 and ADAM17 as major sheddases of Tim-3 as shown by ADAM-specific inhibitors and the ADAM10 prodomain in HEK293 cells and ADAM10/ADAM17-deficient murine embryonic fibroblasts. PMA-induced shedding of Tim-3 was abrogated by deletion of amino acids Glu181–Asp190 of the stalk region and Tim-3 lacking the intracellular domain was not efficiently cleaved after PMA stimulation. Surprisingly, a single lysine residue within the intracellular domain rescues shedding of Tim-3. Shedding of endogenous Tim-3 was found in primary human CD14+ monocytes after PMA and ionomycin stimulation. Importantly, the recently described down-regulation of Tim-3 from Toll-like receptor-activated CD14+ monocytes was caused by ADAM10- and ADAM17-mediated shedding. Inhibition of Tim-3 shedding from lipopolysaccharide-induced monocytes did not influence lipopolysaccharide-induced TNFα and IL-6 but increases IL-12 expression. In summary, we describe Tim-3 as a novel target for ADAM-mediated ectodomain shedding and suggest a role of Tim-3 shedding in TLR-mediated immune responses of CD14+ monocytes.

The T cell immunoglobulin and mucin domain (Tim) family consists of 3 human (Tim-1, -3, and -4) and 4 murine (Tim-1 to -4) Tim proteins (1). Tim-3 is a type I cell-surface glycoprotein with a N-terminal immunoglobulin (Ig)-like domain, a mucin domain with O-linked and N-linked glycosylation, a single transmembrane domain, and a cytoplasmic region with a tyrosine phosphorylation motif (2). Proteins of the Tim family are critically involved in the regulation of immune responses, including allergy, asthma, transplant tolerance, autoimmunity, the response to viral infections, and cancer (1). Galectin-9 is one ligand of Tim-3. Binding of galectin-9 to Tim-3 on T-helper 1 (Th1) and Tc1 cells (a subpopulation of CD8+ cytotoxic T cells) induces cellular apoptosis and Tim-3 deficiency reduces galectin-9-mediated Th1 cell death in vivo (3). In line, down-regulation of Tim-3 allows autoreactive T cells to escape negative regulation in multiple sclerosis (4).

Tim-3 is also highly expressed or up-regulated in exhausted CD8+ T cells in various chronic viral infections (5–8) and in tumor bearing hosts (9–12). Exhausted T cells did not proliferate and fail to exert functions such as cytotoxicity and cytokine secretion in response to antigen stimulation. Interestingly, exhaustion of T cells can be partially overcome by blockade of the interaction of Tim-3 and its ligand. Another hallmark of exhausted T cells is the expression of the inhibitory molecule programmed cell death 1. Like Tim-3, blockade of programmed cell death 1 and programmed cell death 1 ligand (PD-L1) interactions can partially reverse T cell exhaustion (13, 14). Furthermore, blocking of Tim-3 and programmed cell death 1 synergistically restores T cell proliferation, enhances T cell cytokine production, and is effective in restoring anti-tumor immunity in vivo (9). It is, however, not clear whether T cell exhaustion is correlated with interaction of Tim-3 to galectin-9 or another ligand (15).

Tim-3 is also expressed on cells of the innate immune system and can synergize with Toll-like receptors to promote TNFα secretion (16). On CD14+ monocytes, Tim-3 synergizes with TLR signaling to dampen IL-12 secretion with almost no influence on TNFα secretion (17).

Alternative splicing of Tim-3 has been described to result in a hypothetical soluble Tim-3 protein (18). Administration of a human soluble Tim-3-Fc fusion protein caused hyper-proliferation of Th1 cells and Th1 cytokine release and may serve as a
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inhibitor of endogenous Tim-3 function (18). Furthermore, recombinant mouse soluble Tim-3 inhibited T cell responses to antigen-specific stimulation (19). A naturally occurring sTim-3 protein might have antagonistic properties. However, the occurrence of a sTim-3 protein in vivo remains to be shown.

Ectodomain shedding or limited proteolysis of membrane-bound proteins results in protein down-regulation on the cell surface and the production of soluble protein ectodomains with agonistic or antagonistic properties. Members of the A Disintegrin and metalloprotease (ADAM) gene family have emerged as major ectodomain shedding proteases. With more than 100 described substrates so far, ADAM17 and its close relative ADAM10 are the major sheddases of this family (20). There is, however, extensive overlap and compensation between ADAM proteases for several substrates (21, 22). Different stimuli including toxins, bacterial metalloproteinases, and apoptosis activate ADAM10- and/or ADAM17-mediated shedding of transmembrane proteins (20). For some ADAM target proteins such as Notch, induction of intracellular signaling by the remaining intracellular domain cleavage product has been described (23).

Here, we discovered Tim-3 as a novel substrate of ADAM10 and ADAM17, resulting in the release of a soluble Tim-3 protein. A 10-amino acid deletion from Glu181–Asp190 completely abrogated ADAM17-mediated shedding of Tim-3. Whereas deletion of the intracellular domain of Tim-3 largely abrogates PMA-induced shedding, a single lysine residue of the intracellular domain rescued PMA-induced shedding. Finally, down-regulation of Tim-3 from LPS-activated primary human CD14 monoocytes was mediated by ADAM10-mediated shedding.

EXPERIMENTAL PROCEDURES

Cells and Reagents—HEK293 cells were obtained from the American Type Culture Collection (Manassas, VA). Murine embryonic fibroblasts (MEFs) deficient for ADAM10, ADAM17, and ADAM10/ADAM17 were described previously (21, 24–26). All cells were grown in DMEM high glucose culture medium (PAA Laboratories, Cölbe, Germany) supplemented with 10% fetal calf serum (FCS) at 37 °C with 5% CO₂ in a water-saturated atmosphere. Phorbol 12-myristate 13-acetate (PMA) and ionomycin were purchased from Sigma. N-(6-Amino-5-chloro-1-naphthalenesulphonamide) W7 was purchased from Calbiochem, Merck-Millipore, Schwalbach, Germany. The metalloprotease inhibitors GI254023X (GI, ADAM10-selective) and GW280264X (GW, ADAM10- and ADAM17-selective) have been described (27). The recombinant murine pro-domain of the recombinant murine pro-domain of the human hTim-3 (synthesized by GeneScript, Piscataway, NJ) was first digested with HindIII and the AP was subcloned into the pcDNA3.1(+) plasmid. For obtaining the alkaline phosphatase (AP), the plasmid PCRscript-AP (Agilent Technologies, Waldbronn, Germany) was digested with HindIII and the AP was subcloned into the pcDNA3.1-hTim-3 plasmid. The deletions of the sequence coding for the mucin stalk region of the human Tim-3 (from leucine 191 to isoleucine 201 (ΔS1), from glutamic acid 181 to aspartic acid 190 (ΔS2) and asparagine 171 to asparagine 180 (ΔS3)) was performed by splicing by overlap extension (SOE)-PCR, which preserved the original signal peptide coding sequence of the human Tim-3. For the deletion from Leu191 to Ile201 the primers 5′AP-hTim-3-ΔS1f (5′-GCCAATGAGGCATCTACATCGGACG-3′) and 3′AP-hTim-3-ΔS1rev (5′-GCTCCGATGATAGCTCCGCCTATTGCCG-3′) were used. For the deletion from Glu181 to Asp190 the primers 5′AP-hTim-3-ΔS2for (5′-TTGGCAATGACTTACGGGACTCTGG-3′) and 3′AP-hTim-3-ΔS2rev (5′-CCAGATCCGTTAGCTCATTGGCAAT-3′) were used. For the deletion from Asn171 to Asp180 the primers 5′AP-hTim-3-ΔS3for (5′-CCTGATATATAAGTAGTTACGGG-3′) and 3′AP-hTim-3-ΔS3rev (5′-CCGTAGACTCTATTATATACGGGA-3′) were used. The resulting plasmids were named AP-Tim-3ΔS1, AP-Tim-3ΔS2, and AP-Tim-3ΔS3. The deletion mutants of the intracellular domain of Tim-3 (from lysine 225 to leucine 237 (ΔI3), from serine 238 to leucine 250 (ΔI4), and alanine 251 to serine 260 (ΔI5)) were generated by splicing by overlap extension-PCR, the plasmid pcDNA3.1-AP-hTim-3 was used as a template. For the deletion from Lys225 to Leu237 the primers 5′-GATCCCGGCCTTTAATTTTAGCCTCATCTCTTTG-3′ and 3′-AP-hTim-3-ΔI3rev (5′-GATCAGAACAGGAAACAACTGTTAC-3′) were used. For the deletion from Tyr264 to Asp190 the primers 5′-GATCGCCGAGTGACTTTACGGGACTCTGG-3′ and 3′-AP-hTim-3-ΔI4rev (5′-GATCTGCTACTGGTACCTCAGAAGTGGAAT-3′) were used. For the deletion from Trp226 to Asp180 the primers 5′-GATCCCGGCCTTTAATTTTAGCCTCATCTCTTTG-3′ and 3′-AP-hTim-3-ΔI5rev (5′-GATCAGAACAGGAAACAACTGTTAC-3′) were used. For the deletion from Ala251 to Ser260 the primers 5′-GATCGCCGAGTGACTTTACGGGACTCTGG-3′ and 3′-AP-hTim-3-ΔI5rev (5′-GATCTGCTACTGGTACCTCAGAAGTGGAAT-3′) were used. The resulting plasmids were named AP-hTim-3ΔI3, AP-hTim-3ΔI4, and AP-hTim-3ΔI5. The pcDNA3.1-AP-hTim-3 plasmid was used as a template to amplify further deletion mutants of the intracellular domain of Tim-3. For the deletion from Tyr264 to Pro303 the primers 5′-GATCTGTTACCTCAGAAGTGGAATGAC-3′ and 3′-AP-hTim-3-ΔI3rev (5′-GATCAGAACAGGAAACAACTGTTAC-3′) were used. For the deletion from Asn278 to Pro303 the primers 5′-GATCTGCTACTGGTACCTCAGAAGTGGAAT-3′ and 3′-AP-hTim-3-ΔI5rev (5′-GATCAGAACAGGAAACAACTGTTAC-3′) were used. For the deletion from Ser278 to Pro303 the primers 5′-GATCTGCTACTGGTACCTCAGAAGTGGAAT-3′ and 3′-AP-hTim-3-ΔI5rev (5′-GATCAGAACAGGAAACAACTGTTAC-3′) were used. The resulting plasmids were subcloned into pcDNA3.1-AP via AflII and NotI to obtain plasmids pcDNA3.1-AP-hTim-3-ΔI1 and pcDNA3.1-AP-hTim-3-ΔI2, respectively. For the deletion of the whole intracellular domain, the plasmid pcDNA3.1-AP-hTim-3 plasmid was used as a template to amplify the deletion mutant of Tim-3 with primers 5′-GATCGCCGAGTGACTTTACGGGACTCTGG-3′ and 3′-AP-hTim-3-ΔICD (5′-GATCGCCGAGTGACTTTACGGGACTCTGG-3′) were used. The resulting plasmids were subcloned into pcDNA3.1-AP via AflII and NotI to obtain plasmid pcDNA3.1-AP-hTim-3-ΔICD.

For deletion of the intracellular domain starting from Trp226, the pcDNA3.1-AP-hTim-3 plasmid was used as a template to amplify the deletion mutant of Tim-3 with primers 5′-GACTGCGAAGTGGAATGACTCTCAGAAGTGGAAT-3′ and 3′-AP-hTim-3-ΔICD (5′-GACTGCGAAGTGGAATGACTCTCAGAAGTGGAAT-3′) were used. The resulting plasmids were subcloned into pcDNA3.1-AP via AflII and NotI to obtain plasmid pcDNA3.1-AP-hTim-3-ΔICD.
3’) and 3’ hTim-3 (5’-GCTCCGATGTAGATGCCGTCATT-GGC-3’). The resulting PCR products were subcloned into pCDNA3.1-AP via AfII and NotI to obtain plasmid pCDNA3.1-AP-hTim-3-ΔICD+1.

For the deletion of all tags and alkaline phosphatase in plasmid pCDNA3.1-AP-hTim-3 and mutants AP-hTim-3ΔS1, AP-hTim-3ΔS2, and AP-hTim-3ΔS3, the primer 5’ hTim-3-restriction sites. The pET23a-hTim-3-IgV was transformed into the bacterial expression vector pET23a (Novagen, Darmstadt, Germany) using NdeI and NotI. The product was subcloned into the bacterial expression vector pTrc99A (Ampicillin/streptomycin, PAA) and incubated with 5% CO2 at 37 °C. After 2 weeks, the supernatants were pooled and filtered on a 0.22-μm filter and concentrated 10 times. The purification was performed using HiTrap Protein G HP (GE Healthcare) according to the manufacturer’s procedure. Briefly, the column was equilibrated with binding buffer (20 mM phosphate, pH 7.4) followed by loading of the sample. After washing with binding buffer, the mAbs were eluted with 0.1 M glycine, pH 2.7, and directly neutralized with 1 M Tris-HCl, pH 9.0. The buffer was changed by NAP-10 column (GE Healthcare) to PBS. The purity of the mAbs was verified by SDS-PAGE (data not shown).

Transfection of Cells—HEK293 cells and MEFs were transiently transfected using TurboFect (ThermoFisherScientific Inc., Waltham, MA).

Western Blotting—For immunochemical detection of Tim-3 proteins, cells were washed three times with sterile PBS. Inhibitors were added 30 min before stimulation with PMA, ionomycin, or W7. Stimulation was performed in serum-free DMEM. Subsequently, cells were centrifuged and the pellet was directly frozen in liquid nitrogen. Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5 mM EDTA, and 0.5% IGEPAL (Nonidet P-40), supplemented with complete protease inhibitor mixture tablets (Roche Diagnostics). The conditioned media were concentrated to final volume of 300 μl by SpeedVac® Plus SC110A (ThermoScientific, Dreieich, Germany). Proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Whatman-Fisher Scientific GmbH, Schwerte, Germany). The membrane was blocked with 6% skimmed milk in Tris-buffered saline (TBS-T; 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, and 0.05% Tween 20 (TBS-T; 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.5% Tween 20) and probed with primary antibodies as indicated at 4 °C overnight. After washing with TBS-T, the membranes were incubated with the appropriate secondary antibodies conjugated to horseradish peroxidase (HRP; Thermo Scientific/ Pierce, Perbio, Bonn, Germany), and protein bands were visualized with the Pierce ECL Western blotting substrates (Thermo Scientific Inc., Rockford, IL) according to the manufacturer’s instructions.

Phosphatase Assay—For detection of AP-Tim-3 proteins, cells were treated as described for Western blotting. The conditioned media were collected and directly frozen in liquid nitrogen. For alkaline phosphatase activity measurements, 50 μl of reaction solution (0.1 μl of 1 mg/ml of 4-nitrophenyl phosphate disodium salt hexahydrate, Sigma) were added to 50 μl of the conditioned medium. The alkaline phosphatase removes the phosphate group from the p-nitrophenyl phosphate, which is then deprotonated under alkaline conditions to produce p-nitrophenolate which has a strong absorption at 405 nm. So, the absorbance was read at 405 nm and refers to the alkaline phosphatase activity. The absorbance of the AP-hTim-3 proteins in the supernatant was normalized to the transfection efficiency of each sample, by division of the total absorbance of the transfected cells (sum of the absorbance of the supernatant and the lysate).
Calmodulin Pull-down Assay—For pull-down assays HEK293 cells were transfected with the expression plasmids coding for AP-Tim-3, AP-Tim-3 deletion mutant (AP-Tim-3ΔS2), AP-Tim-3 lacking the intracellular domain (ΔICD), or AP-Tim-3 without the intracellular domain with the intracellular lysine at position 226 (ΔICD+1) fusion proteins. Transfected HEK293...
cells were lysed with 20 mM HEPES, 200 mM KCl, 0.55% Triton X-100, pH 7.4, and centrifuged at 15,000 × g for 10 min to remove insoluble material. Lysates were incubated with 100 µl of calmodulin-Sepharose 4B or Sepharose 4B (GE Healthcare) in calmodulin buffer (20 mM HEPES, 200 mM KCl, pH 7.4) overnight at 4 °C. After extensive washing, immobilized proteins were resuspended in 50 µl of SDS sample buffer (4 times).

**Isolation and Cultivation of Human CD14⁺ Monocytes**—Human peripheral blood mononuclear cells (PBMC) were isolated from peripheral blood using lymphocyte separation medium LSM 1077 (PAA) density gradient centrifugation. CD14⁺ monocytes were further purified from PBMCs by negative selection using monocyte Isolation Kit II with column purification following the manufacturer’s instructions (Miltenyi Biotec, Bergisch-Gladbach, Germany). The cells were cultured in RPMI 1640 containing 10% FCS, 100 µg/ml of penicillin-streptomycin, and 2 mM l-glutamine (PAA). All patients underwent a written, informed-consent process approved by the ethics commission of the Medical Faculty of Kiel University (AD 404/12).

**Flow Cytometry Staining and Analysis**—PBMCs from 3 to 5 healthy subjects were stimulated with 5 µg/ml of LPS (Invitrogen) for various time points as indicated. Cell-surface staining was carried out using anti-Tim-3-PE clone F38-2E2 (Biolegend, London, Great Britain) or isotype-matched control antibodies (BD Biosciences) to determine the levels of background staining. The cells were analyzed by flow cytometry (BD Biosciences; FACSCanto and FACS DIVA software).

To detect the surface expression of hTim-3 and mutants thereof, transiently transfected HEK293 cells were washed with FACS buffer (PBS, 0.25% bovine serum albumin (BSA) and incubated at 5 × 10⁵ cells/100 µl of FACS buffer containing 1:20 diluted PE-anti-human Tim-3, clone F38-2E2 (Biolegend) or PE-mouse IgG1, κ isotype control in FACS buffer for 60 min at 4 °C. Cells were washed once with FACS buffer, re-suspended, and analyzed by flow cytometry (BD Biosciences; FACSCanto and FACS DIVA software). For the combination of different experiments, the amount of Tim-3 expressing cells was set to 100% and loss of Tim-3 was calculated.

**ELISA**—Cytokine levels were measured via ELISA using DuoSet ELISA kits (R&D Systems).

**RESULTS**

**ADAM10 and ADAM17 Are the Major Sheddases of Tim-3**—Tim-3 is a type I membrane protein and consists of a N-terminal Ig-like domain, a highly glycosylated mucin stalk, a single transmembrane domain, and a cytoplasmic region. HEK293 cells were transfected with an expression plasmid coding for AP-tagged Tim-3. AP-Tim-3 cell-surface expression was verified by flow cytometry using an anti-Tim-3 mAb in HEK293 cells (Fig. 1A). To test whether TIM-3 is a substrate for ADAM proteases, AP-Tim-3 expressing HEK293 cells were stimulated with PMA or ionomycin in the absence or presence of the ADAM10 inhibitor GI254023X (GI) and ADAM10/ADAM17 inhibitor GW280264X (GW) or left untreated. Ionomycin and benzyl-ATP are activators of ADAM10-mediated ectodomain shedding and PMA is a general activator of ADAM17-mediated shedding (23). PMA and ionomycin treatment of AP-Tim-3 expressing HEK293 cells led to the release of soluble AP-Tim-3 into the cell culture supernatant as quantified by an alkaline phosphatase assay (Fig. 1B). The combined ADAM10/ADAM17 inhibitor GW but not the ADAM10 inhibitor GI completely inhibited the PMA-induced and constitutive release of AP-Tim-3 into the cell culture supernatant. Ionomycin-induced shedding was inhibited by both, the ADAM10 inhibitor GI and the ADAM10/ADAM17 inhibitor GW (Fig. 1B). The presence of a soluble AP-Tim-3 protein with the expected size was detected by Western blotting using anti-Tim-3 mAb directed against the Ig-like domain. Again PMA-induced shedding was only inhibited by GW, whereas ionomycin-induced shedding was inhibited by GI and GW (Fig. 1C). Next, we inhibited ADAM10 with the recombinant pro-domain of ADAM10 (proA10) (28) in PMA- and ionomycin-treated HEK293 cells, transiently transfected with AP-Tim-3 cDNA. As expected, proA10 inhibits ionomycin-induced AP-Tim-3 shedding. However, it appears also that PMA-induced shedding of AP-Tim-3 was partly inhibited by proA10 (Fig. 1, D and E), which might be due to partial blocking of ADAM17 (29). We have, however, not observed cross-inhibition with proADAM10 of ADAM17-mediated shedding of IL-6R (21). MEFs deficient for ADAM10, ADAM17, and ADAM10/ADAM17 were transiently transfected with the cDNA coding for AP-Tim-3. As depicted in Fig. 1, F and G, shedding of AP-Tim-3 was induced by PMA and ionomycin treatment in wild-type MEFs. PMA-induced shedding was, however, completely abrogated in ADAM17-deficient MEFs but only minimally affected in ADAM10-deficient MEFs. Ionomycin-induced shedding was almost completely inhibited in ADAM10-deficient MEFs but not in ADAM17-deficient MEFs. Shedding of Tim-3 was completely inhibited in

**FIGURE 1. Analysis of human Tim-3 for ionomycin- and PMA-induced shedding by ADAM metalloproteinases.** A, expression of the AP-Tim-3 fusion protein on the cell surface of HEK293 cells. HEK293 cells were transfected with the expression plasmid for the AP-Tim-3 fusion protein and immunostained for Tim-3 using anti-Tim-3-PE clone F38-2E2. B and C, HEK293 cells were transfected with expression plasmid encoding the AP-Tim-3 and treated for 1 h with PMA (100 nM) or 30 min with ionomycin (5 µM). Gl (3 µM) or GW (3 µM) were added 30 min prior to stimulation. D and E, inhibitory recombinant ADAM10 pro-domain (A10pro, 10 µM) was added 30 min prior to stimulation with PMA or ionomycin. The alkaline phosphatase activity was measured in the lysate and the conditioned medium. AP activity is calculated as ratio of the alkaline phosphatase activity in the lysate and supernatant. C and D, HEK293 cells were treated as described in panels B and C. Membrane-bound Tim-3 in the cell lysates and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”) and β-actin mAbs (Cell Signaling). F and G, MEFs deficient for ADAM10, ADAM17, or for both ADAM10 and ADAM17 and wild-type MEFs were transfected with the expression plasmid for the AP-Tim-3 and stimulated as indicated. Tim-3 levels in the lysates and the conditioned medium was quantified by AP assay and visualized by Western blotting as described in C, H, expression of the human Tim-3 in HEK293 cells. HEK293 cells were stably transfected with the expression plasmid of Tim-3 and immunostained for TIM-3 using anti-Tim-3-PE clone F38-2E2. HEK293 cells were stably transfected with expression plasmid encoding Tim-3 and treated for 1 h with PMA (100 nM) or 30 min with ionomycin (5 µM). Gl (3 µM) or GW (3 µM) were added 30 min prior to stimulation. Inhibitory recombinant ADAM10 pro-domain (A10pro, 10 µM) was added 30 min prior to stimulation with PMA or ionomycin. Membrane-bound Tim-3 in the cell lysates (I and J) and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”). The values are from one representative experiment (n = 5). n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001 (unpaired Student's t-tests).
ADAM10/ADAM17 double-deficient MEFs after PMA and ionomycin treatment.

To exclude that the shedding results obtained with AP-tagged Tim-3 were influenced by the AP tag, we removed the AP tag from the AP-Tim-3 cDNA. N terminally FLAG-tagged human Tim-3 was stably transfected in HEK293 cells, and cell-surface expression of Tim-3 was verified by flow cytometry (Fig. 1H). Shedding of Tim-3 was verified by Western blotting against
Tim-3 in cell lysates and supernatants. As AP-tagged Tim-3, Tim-3 was shed after PMA and ionomycin treatment from HEK293 cells (Fig. 1). Again, PMA-induced shedding of Tim-3 was inhibited by GW but to a much lower extend by GI or proA10 (Fig. 1, I and J). Ionomycin-induced shedding of Tim-3 was inhibited by GI, GW, and proA10 (Fig. 1, I and J). Taken together, these results indicated that Tim-3 is a substrate of ADAM proteases. Whereas ionomycin-induced ADAM110-mediated Tim-3 shedding, PMA-induced ADAM17-mediated shedding of Tim-3. Our inhibitor studies but not the data obtained from gene-deficient MEFs indicate that ADAM10 might also play a minor role in PMA-induced shedding of Tim-3.

PMA-induced Shedding of Tim-3 Was Abrogated by Deletion of 10 Amino Acids from the Stalk Region—Shedding of cell-surface proteins by ADAM proteases is described to occur in close juxta-position to the plasma membrane (23). For instance, the ADAM17-cleavage site for the interleukin-6 receptor, a substrate for ADAM10 and ADAM17, is located between Gln357 and Asp358 about 8 amino acids away from the plasma membrane, and deletion of 10 amino acids surrounding the cleavage site (Ser153–Val362) completely inhibited PMA-induced shedding by ADAM17 (30) but not of ADAM10 (31). The prediction of ADAM cleavage sites is complicated by the fact that no clear consensus cleavage sequence exists in substrates of ADAM10 and ADAM17. A tendency, however, for smaller aliphatic residues at P1 (Val, Ala) for ADAM17 and ADAM10 is eminent (32). This illustrates why it is not possible to predict ADAM10 and ADAM17 substrates using sequence analysis. The stalk of Tim-3 compromises 70 amino acids from Ala132 to Ile201. Here, we generated 3 different variants of Tim-3 (ΔS1, ΔS2, ΔS1–181; ΔS3, ΔS1–171) each lacking 10 amino acids prior to the transmembrane domain (Fig. 2A). As shown in Fig. 2B, all three Tim-3 deletion variants were expressed on the cell surface of transfected HEK293 cells. PMA-induced shedding was, however, completely abrogated for AP-Tim-3ΔS2 but not affected in AP-Tim-3ΔS1 and AP-Tim-3ΔS3 (Fig. 2, C and D). Again, PMA fusion did not influence the shedding behavior of Tim-3 (Fig. 2E). Ionomycin-induced shedding was not changed for any of the three Tim-3 deletion variants (Fig. 2, C–E). Although, we have not determined the cleavage site(s) for ADAM17 directly, we conclude from these experiments that the cleavage site for PMA-induced proteolysis of Tim-3 (mediated by ADAM17) is located between amino acid positions 181 and 190. Alternatively, recognition of Tim-3 by ADAM17 might be regulated by this sequence. Our results also illustrate that the cleavage sites for ADAM10 and ADAM17 in Tim-3 are different. Our results open up the possibility to generate ADAM17-uncleavable knock-in mice for Tim-3.

The Intracellular Domain of Tim-3 Is Important for PMA-induced Shedding—PMA-induced shedding of L-selectin was dependent on the interaction with the actin cytoskeleton via ezrin-radixin-moesin (ERM) proteins (33). Thereby, L-selectin is the only example where a role of the intracellular domain in ectodomain shedding was assigned. HEK293 cells were transiently transfected with an AP-Tim-3 cDNA lacking the coding sequence of the complete intracellular domain from Lys225 to Gln301 (AP-Tim-3ΔICD) (Fig. 3A), which was expressed on the cell surface of HEK293 cells (Fig. 3B). Whereas AP-Tim-3 was shed after PMA or ionomycin treatment as described above, shedding of the AP-Tim-3ΔICD was not induced by PMA but remained intact after ionomycin treatment as shown by AP assays and Western blotting (Fig. 3, C and D). Again, PMA fusion did not influence the shedding behavior of Tim-3 (Fig. 3D). To analyze whether phosphorylation of the intracellular domain of Tim-3 might be involved in PMA-induced shedding, two additional deletion variants and AP-Tim-3ΔA278–301 (ΔI1) and AP-Tim-3ΔA264–301 (ΔI2) were generated lacking both or only the C-terminally located tyrosine phosphorylation site (34) (Fig. 3A). PMA- and ionomycin-induced shedding was, however, not disturbed in AP-Tim-3ΔI1 and AP-Tim-3ΔI2 as shown by AP assay and Western blotting (Fig. 3, E and F), indicating that tyrosine phosphorylation is not involved in PMA-induced shedding. Therefore, we generated three additional Tim-3 deletion variants, each lacking AP-Tim-3ΔA225–237 (ΔI3), AP-Tim-3ΔA238–250 (ΔI4), and AP-Tim-3ΔA251–260 (ΔI5), which contain deletions spanning the remaining ICD of Tim-3 (Fig. 3A). However, none of these deletions led to abrogation of PMA-induced shedding (Fig. 3, G and H). Finally, we generated the deletion variant AP-Tim-3ΔW226-Gln301 (ΔICD+1) (Fig. 3A), which differs from AP-Tim-3ΔICD by the addition of the first intracellular residue Lys225. Surprisingly, PMA-induced shedding of AP-Tim-3ΔICD+1 was also indistinguishable from full-length Tim-3 (Fig. 3, I and J).

Calmodulin Contributes to Tim-3 Cell Surface Expression—The proteolytic processing of Tim-3 shares some common feature with the shedding of L-selectin. The expression of L-selectin is rapidly down-regulated upon cell activation through proteolysis at a membrane-proximal site (35). The calcium regulatory protein calmodulin (CaM) is directly associated with the cytoplasmic and transmembrane domains of L-selectin. Calmodulin antagonists including N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) induce the proteolytic release

FIGURE 2. PMA-induced shedding of Tim-3 was abrogated by deletion of 10 amino acids from the stalk region. A, schematic overview of the AP-Tim-3 constructs used in this study. Three constructs with three different deletions of 10 amino acids each from the mucin stalk region (AP-Tim-3ΔS1 to AP-Tim-3ΔS3) were created as described. All constructs contain the alkaline phosphatase at the N terminus. B, HEK293 cells were transfected with the expression plasmids for AP-Tim-3 deletion mutants (AP-Tim-3ΔS1 to AP-Tim-3ΔS3) and immunostained for AP-Tim-3 using anti-Tim-3-PE clone F38–2E2. C, HEK293 cells were transfected with expression plasmids encoding AP-Tim-3 or the deletion mutants (AP-Tim-3ΔS1 to AP-Tim-3ΔS3) and treated for 1 h with PMA (100 nM) or 30 min with ionomycin (5 μM). The alkaline phosphatase activity was measured in the lysate and conditioned medium. The AP activity is calculated as the ratio of the alkaline phosphatase activity in the lysate and supernatant. D, membrane-bound Tim-3 in the cell lysates and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAbs (description under “Experimental Procedures”). The values are from one representative experiment (n = 5). E, HEK293 cells were transfected with an expression plasmid encoding Tim-3 deletion mutants (Tim-3ΔS1 to Tim-3ΔS3) without alkaline phosphatase at the N terminus and treated for 1 h with PMA (100 nM) or 30 min with ionomycin (5 μM). Membrane-bound Tim-3 in the cell lysates and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”). The values are from one representative experiment (n = 5). n.s., not significant; **, p < 0.01; ***, p < 0.001 (unpaired Student’s t tests).
of L-selectin from the cell surface. These effects can be prevented by co-treatment with hydroxamic acid-based metallopeptase inhibitors. The binding of CaM also to other proteins such as collagen receptors or amyloid precursor protein prevents shedding and disruption of these interactions result in the activation of ADAM-mediated ectodomain shedding (36).

HEK293 cells expressing AP-Tim-3 were stimulated with W7 in the absence or presence of GI and GW or left untreated. AP-Tim-3 protein released into the supernatant was quantified by AP assay. W7 treatment led to the release of soluble AP-Tim-3 into the cell culture supernatant as shown by the AP assay and Western blotting (Fig. 4, A and B). The ADAM10

![Diagram](image)
inhibitor GI and combined ADAM10/ADAM17 inhibitor GW prevented the W7-induced release of AP-Tim-3 into the cell culture supernatant (Fig. 4, A and B), with GW having a stronger effect.

Next, we investigated if the PMA-resistant Tim-3 variant AP-Tim-3ΔS2 is proteolytically cleaved after W7 treatment. As expected, the W7-induced shedding was also nearly completely abrogated for AP-Tim-3ΔS2 as shown before for PMA-induced cleavage (Fig. 4, C and D). Taken together, these data indicated that W7 induced ADAM10- and ADAM17-mediated shedding of Tim-3.

CaM binds to the membrane-proximal region of the cytoplasmic domain plus a small region of the membrane-spanning domain of L-selectin (37). To analyze if the CaM-binding site of Tim-3 is also located in the intracellular domain of Tim-3, we used the AP-Tim-3 cDNA lacking the coding sequence of the complete intracellular domain from Lys225 to Gln301 (AP-Tim-3ΔICD) and the deletion variant AP-Tim-3ΔW226-Gln301 (ΔICD+1), which differs from AP-Tim-3ΔICD by the addition of the first intracellular residue Lys225. Shedding of AP-Tim-3ΔICD was strongly diminished in comparison to the AP-Tim-3, whereas the deletion variant AP-Tim-3ΔW226-Gln301 (ΔICD+1) was shed upon CaM inhibition, which was reduced by GI and GW (Fig. 4, E–G).

To exclude that results obtained with AP-tagged Tim-3 were influenced by the AP tag, we used HEK293 cells transiently transfected with Tim-3. Shedding of Tim-3 was verified by Western blotting against Tim-3 in cell lysates and supernatants. As AP-tagged Tim-3, Tim-3 was shed after W7 treatment from HEK293 cells (Fig. 4H). Again, W7-induced shedding of Tim-3 was inhibited by GW and GI, albeit to a lower extent (Fig. 4, A and F). Shedding of Tim-3ΔS2 and Tim-3ΔICD was also comparable with the AP-tagged variants in transfected HEK293 cells (Fig. 4, A and F). Finally, we showed that CaM was precipitated in a complex with Tim-3, indicating that CaM interacts with Tim-3 but also with all deletion mutants (Fig. 4I). The finding that the intracellular domain of Tim-3 was negligible for calmodulin-prevented shedding was surprising. It has been recently shown that calmodulin not only binds to residues within the intracellular domain but also to areas within the transmembrane domain (34). Therefore calmodulin might also interact with the intracellular domain of Tim-3. We cannot, however, exclude at the moment, that calmodulin is interacting with another protein involved in regulation of ADAM-mediated shedding of Tim-3, which might be part of a higher order Tim-3 protein complex. Taken together, these results show that ADAM-mediated shedding of Tim-3 is CaM-dependent and mediated by ADAM10 and -17.

PMA- and Ionomycin-induced Shedding of Endogenous Tim-3 in Primary Human CD14+ Monocytes—To analyze if endogenous Tim-3 is also a substrate of ADAM-mediated shedding, we used CD14+ monocytes isolated from PBMCs, which naturally express Tim-3 (17). CD14+ monocytes also express ADAM10 and ADAM17 on the cell surface (data not shown). At least 49% (varies between 49 and 77% from experiment to experiment) of the isolated CD14+ monocytes expressed Tim-3 on the cell surface as judged by flow cytometry (Fig. 5). Ionomycin treatment led to a drastic reduction of Tim-3 cell-surface expression and only about 7.4% of the cells showed cell-surface expression of Tim-3. Importantly, ionomycin-induced loss of cell surface Tim-3 was prevented by co-incubation with GI or GW (Fig. 5, A and B), indicating that the loss of cell surface Tim-3 was due to ADAM10-mediated shedding of Tim-3. Albeit to a lesser extent, PMA treatment led to a reduction of Tim-3 from the cell surface of CD14+ monocytes and only about 30.9% (untreated 64%) of the cells showed cell-surface expression of Tim-3. Loss of cell surface Tim-3 was rescued by co-incubation with the combined ADAM10 and ADAM17 inhibitor GW (50.6%) and to a lesser degree by the ADAM10 inhibitor GI (42.7%) (Fig. 5, C and D). Taken together, endogenous Tim-3 is a substrate of ADAM10 after ionomycin treatment and preferentially a substrate of ADAM17 after PMA treatment. However, our data again indicate that ADAM10 might also be involved in PMA-induced shedding of Tim-3.

Shedding of Tim-3 from Toll-like Receptor (TLR)-activated CD14+ Monocytes Was Caused by ADAM10-mediated Shedding—LPS leads to TLR-mediated activation of CD14+ monocytes, and expression of cytokines including IL-6, TNFa, and IL-12. Recently, rapid down-regulation of Tim-3 from the cell surface of LPS-activated CD14+ monocytes was linked to up-regulation of IL-6 and IL-12 (17). Blockade of Tim-3 signaling or silencing of Tim-3 led to a significant acceleration of LPS-mediated IL-6 and IL-12 expression (17), indicating that Tim-3 regulates innate immune responses in CD14+ monocytes. Here, we analyzed if down-regulation of Tim-3 on CD14+ monocytes after LPS stimulation was caused by shedding and whether Tim-3 shedding was functionally linked to cytokine

FIGURE 3. Tim-3 lacking the intracellular domain is not cleaved after PMA treatment. A, amino acid sequence of human Tim-3. Truncation mutants are marked as ΔI1, ΔI2, ΔI3, ΔI4, and ΔI5. Deletion mutants in the intracellular domain of 10 amino acids each are indicated as ΔI1ΔI5. B, HEK293 cells were transfected with the expression plasmid for the AP-Tim-3 without the intracellular domain (ΔICD) fusion protein and immunostained for Tim-3. ICD in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”). The values are from one representative experiment (n = 3). F and G, HEK293 cells were transfected with expression plasmids encoding the AP-Tim-3 with the indicated truncation (ΔI1 and ΔI2). G and H, HEK293 cells were transfected with the expression plasmid encoding the AP fusion protein Tim-3 with the indicated truncation (ΔI3ΔI5). For AP activity and Western blotting see C and D. The values are from one representative experiment (n = 3), I, HEK293 cells were transfected with the expression plasmid for the AP-Tim-3 without the intracellular domain with the indicated intracellular lysine at position 226 (ΔICD+1) fusion protein and treated for 1 h with PMA (100 μM) or 30 min with ionomycin (5 μM). The alkaline phosphatase activity was measured in the lysate and conditioned medium. The AP activity was calculated as the ratio of the alkaline phosphatase activity in the lysate and conditioned medium. The values are from one representative experiment (n = 3). F and G, HEK293 cells were transfected with expression plasmids encoding the AP-Tim-3 with the indicated truncation (ΔI1 and ΔI2). G and H, HEK293 cells were transfected with the expression plasmid encoding the AP fusion protein Tim-3 with the indicated truncation (ΔI3ΔI5). For AP activity and Western blotting see C and D. The values are from one representative experiment (n = 3), I, n.s., not significant; **, p < 0.01; ***, p < 0.001 (unpaired Student’s t tests).
expression. Primary human CD14⁺ monocytes were stimulated with LPS and cell-surface expressed Tim-3 was quantified by flow cytometry. Again, CD14⁺ monocytes expressed high levels of Tim-3, with about 77.4% of the cells expressing Tim-3 on the cell surface. After 2 h of LPS stimulation, only about 35.4% of the monocytes expressed Tim-3 on the cell surface.
Importantly, Tim-3 down-regulation was prevented by treatment with GI (57.8%), GW (71.6%), and proA10 (66.4%), indicating that ADAM10 and ADAM17 were activated after LPS stimulation to shed Tim-3 from the cell surface in a time-dependent manner (Fig. 5, E–G). Next, we tested if shedding of Tim-3 negatively regulates TNFα, IL-6, and IL-12 expression. Therefore, CD14+ monocytes were stimulated with LPS for 24 h. Shedding of Tim-3 was prevented by addition of GI and GW. Subsequently, TNFα, IL-6, and IL-12 were quantified after 2, 4, 6, 12, 18, and 24 h in the cell culture supernatants. As expected, LPS stimulation increases the concentrations of IL-6, TNFα, and IL-12 (Fig. 6, A–C). If shedding of Tim-3 would negatively regulate IL-6 and IL-12 cytokine expression, than we would expect that inhibition of shedding would lead to a decrease of IL-6 and IL-12 cytokine production. However, abrogation of LPS-induced shedding by GI and GW has no influence on the accumulation of TNFα and IL-6 and IL-12 expression. Interestingly, GI and GW led to an increase of IL-12 after LPS stimulation (Fig. 6C). We have to admit that the up-regulation of IL-12 was not necessarily functionally connected to inhibition of Tim-3 shedding, because the inhibitors generally inhibit ADAM10 and ADAM17. Final proof, however, cannot be achieved with inhibition of ADAM proteases but with a selective inhibition of Tim-3 shedding, as we have shown for Tim-3ΔS2 and Tim-3ICD. Therefore, the generation of Tim-3 uncleavable knock-in or transgenic mice would be desirable to explore the function of Tim-3 shedding during immune responses.

**DISCUSSION**

Here, we identified Tim-3 as a novel substrate of ADAM10- and ADAM17-mediated ectodomain shedding. Shedding of Tim-3 from HEK293 cells was induced after stimulation with ionomycin or PMA. Ionomycin-induced shedding was inhibited by ADAM10- and ADAM10/ADAM17-selective inhibitors GI and GW and the ADAM10 pro-domain. PMA-induced shedding was inhibited by the ADAM10/ADAM17-selective inhibitor GW and to a lesser extent by GI and the ADAM10 pro-domain. These results indicate that PMA-induced shedding is mainly mediated by ADAM17 and in part by ADAM10, whereas ionomycin-induced shedding is solely mediated by ADAM10. These results were verified using ADAM10- and/or ADAM17-deficient murine embryonic fibroblasts. Here, we used a self-made anti-Tim-3 mAb to detect Tim-3 by Western blotting. To verify Tim-3 shedding, we also used the detection of AP-tagged Tim-3, which was described previously (38).

ADAM10 or ADAM17 knock-out mice are embryonically lethal (25, 39), which underlines the physiological importance of these two proteases. Conditional knock-out mice have highlighted the importance of ADAM10 and ADAM17 in various physiological conditions and inhibition of one or both proteases is still considered a therapeutic option (40–42). ADAM10 and ADAM17 have, however, more than 100 substrates (23), some are exclusively shed by ADAM10 or ADAM17 and some are shared substrates. Importantly, no defined consensus cleavage sequences exists for ADAM10 and ADAM17 (32). Moreover, substrate recognition and regulation of selective substrate cleavage by ADAM proteases is largely unknown. However, recently, iRhom2 was reported to facilitate ADAM17 trafficking and maturation. Consequently, iRhom2-deficient mice fail to shed TNF (43, 44). Moreover, iRhom1 and iRhom2 were shown to be involved in substrate recognition by ADAM17 (45).

For some substrates including TNFα, EGF, and Notch, ADAM-mediated cleavage has been validated in vivo and the relevance of the cleavage of most substrates is still unknown. Genetic alteration of the ADAM cleavage site of a single substrate seems to be a promising strategy for in vivo analysis of defective shedding of a single substrate. Genetically modified mice encoding an ADAM17 shedding-resistant L-selectin variant showed a drastically reduced serum level of soluble L-selectin and increased cell-surface expression (46, 47). Using deletion variants of the stalk domain, we identified an ADAM17 un-cleavable Tim-3 variant (Tim3ΔS2), which after translation into murine Tim-3 enables the generation of an un-cleavable murine Tim-3 knock-in mice to study the in vivo relevance of Tim-3 shedding in mice. Moreover, we speculate that the cleavage site of ADAM17 in Tim-3 is located between Glu181 and Asp (91) (Tim3ΔS2).

The intracellular domain of Tim-3 also contributes to ADAM17- but not to ADAM10-mediated shedding of Tim-3.

**FIGURE 4. Tim-3 proteolysis is regulated by calmodulin.** HEK293 cells were transfected with the expression plasmids for AP-Tim-3, AP-Tim-3 deletion mutant (AP-Tim-3ΔS2), AP-Tim-3 lacking the intracellular domain (ΔICD) or AP-Tim-3 without the intracellular domain with the intracellular lysine at position 226 (ΔICD+L). HEK293 cells were transfected with expression plasmids for AP-Tim-3, AP-Tim-3 deletion mutant (AP-Tim-3ΔS2), AP-Tim-3 lacking the intracellular domain (ΔICD), or AP-Tim-3 without the intracellular domain with the intracellular lysine at position 226 (ΔICD+L) fusion proteins. Transfected HEK293 cells were incubated in the absence or presence of GI or GW (3 μM, 30 min prior to stimulation) as indicated. The AP activity was calculated as the ratio of alkaline phosphatase activity in the lystate and the conditioned medium (A, E, and F). B, D, and G, membrane-bound AP-Tim-3 in cell lysates and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using the anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”). H, HEK293 cells were transfected with expression plasmid encoding Tim-3 or Tim-3 deletion mutants Tim-3ΔS2 or Tim-3ICD without alkaline phosphatase at the N-terminus and treated for 1 h with W7 (100 μM). The transfected HEK293 cells were incubated in the absence or presence of GI or GW (3 μM, 30 min prior to stimulation) as indicated. Membrane-bound Tim-3 in the cell lysates and soluble Tim-3 in the conditioned medium were subsequently visualized by Western blotting using anti-hTim-3 IgV-domain mAbs (description under “Experimental Procedures”). The values are from one representative experiment (n = 3). n.s., not significant; *, p < 0.05; **, p < 0.01; ***, p < 0.001 (Student’s t tests). J, HEK293 cells were transfected with expression plasmids for AP-Tim-3, AP-Tim-3 deletion mutant (AP-Tim-3ΔS2), AP-Tim-3 lacking the intracellular domain (ΔICD), or AP-Tim-3 without the intracellular domain with the intracellular lysine at position 226 (ΔICD+L) fusion proteins. Transfected HEK293 cells were lysed and incubated with calmodulin-Sepharose 4B (Cam-Sepharose) or an equivalent amount of Sepharose 4B (Sepharose). Proteins precipitated by immobilized calmodulin were separated on a 15% acrylamide gel, and probed with the presence of Tim-3 by immunoblotting with anti-hTim-3 IgV-domain mAb (description under “Experimental Procedures”).
FIGURE 5. Expression of endogenous Tim-3 upon PMA, ionomycin, or LPS stimulation on human primary CD14⁺ monocytes. A–D, decreased Tim-3 expression on negatively selected CD14⁺ M/M upon stimulation with ionomycin or PMA. CD14⁺ monocytes isolated from healthy subjects were treated for 1 h with PMA (100 nM) or 30 min with ionomycin (1 μM). GI (9 μM) or GW (9 μM) were added 30 min prior to stimulation, followed by immunostaining and flow cytometric analysis for the expression of CD14 and Tim-3. Cells were first gated on the monocytic population and then analyzed for the percentage of Tim-3 positive cells in the CD14⁺ cell population. B and D, summary data (mean ± S.D. from four healthy subjects) of percentages of Tim-3 positive cells are shown. *, p < 0.05; ***, p < 0.001 (unpaired Student’s t tests).

E and F, decreased Tim-3 expression on positively selected CD14⁺ M/M upon TLR stimulation. This effect was restored by specific inhibition of ADAM10 and ADAM17. CD14⁺ monocytes isolated from healthy subjects were treated for 2 h with LPS (5 μg/ml). GI (9 μM) or GW (9 μM) were added 30 min prior to stimulation. Inhibitory recombinant ADAM10 pro-domain (A10pro, 10 μM) was also added 30 min prior to stimulation or without 5 μg/ml of LPS for 2 h, followed by immunostaining and flow cytometric analysis for the expression of CD14 and Tim-3. Cells were first gated on the monocytic population and then analyzed for the percentage of Tim-3 positive cells in the CD14⁺ cell population. F, summary data (mean ± S.D. from four healthy subjects) of percentages of Tim-3 positive cells are shown after 2 h of TLR stimulation. **, p < 0.01; ***, p < 0.001 (unpaired Student’s t tests).

G, kinetics of Tim-3 expression in CD14⁺ M/M following TLR stimulation. CD14⁺ monocytes from three healthy subjects were incubated with or without TLR ligand LPS for various time points, followed by immunostaining and flow cytometry analysis for the expression of CD14 and Tim-3. The percentage of Tim-3 CD14⁺ M/M at different time points is calculated, and the mean ± S.D. of the double positive cell from these healthy subjects is shown. **, p < 0.01; ***, p < 0.001 (unpaired Student’s t tests).
because deletion of the complete intracellular domain abrogates PMA- but not ionomycin-induced shedding (Tim-3ΔICD). To identify the region within the intracellular domain that contributes to PMA-induced shedding, we generated and tested additional intracellular deletion variants. Truncation 1 (ΔI1) lacks all but the three tyrosines, whereas truncation 2 (ΔI2) comprises all but one tyrosine, which resides close to the membrane. Tyrosines 256 and 263, which are deleted in Tim-3ΔI2, were shown to be phosphorylated and to enhance NFAT/AP-1 activation (34). Shedding of Tim-3ΔI1 and Tim-3ΔI2 was, however, indistinguishable from wild-type Tim-3. Also the three deletion variants Tim-3ΔI3, -ΔI4, and -ΔI5, comprising deletions of 9–12 amino acids from Lys225 to Ser260, were shed like wild-type Tim-3 after PMA stimulation. These results were unexpected and we decided to generate the Tim-3 deletion variant Tim-3ΔICD + 1, which covers a deletion of the intracellular domain from Trp226 to Pro301. Surprisingly, the single intracellular lysine residue was sufficient to rescue PMA-induced shedding. At the moment, we have not investigated the mechanism for this phenomenon. To the best of our knowledge, this is the first example that ADAM17-mediated shedding is regulated by the presence or absence of a single intracellular amino acid. For most substrates, involvement of the intracellular domain for ADAM-mediated shedding was not investigated. In the case of the IL-6R, deletion of the intracellular domain did not influence PMA-induced shedding (30). For L-selectin, the intracellular

FIGURE 6. Kinetics of TNF-α, IL-6, and IL-12 production in positively selected CD14+ M/M from healthy subjects stimulated with LPS. CD14+ monocytes isolated from healthy subjects were treated for 2 h with LPS (5 μg/ml), followed by ELISA of the cell culture supernatant. The values are from one representative experiment (n = 4).
domain participates in regulation of shedding. Here, CaM is constitutively associated with the intracellular membrane-proximal region of L-selectin and abrogation of calmodulin binding increases the proteolytic turnover of L-selectin (48). Here, we show that CaM is also interacting with Tim-3 and inhibition of CaM binding to Tim-3 by the inhibitor W7 induces ADAM17-mediated shedding of Tim-3. Shedding also requires anchoring of L-selectin to the cytoskeleton via members of the ezrin-radixin-moesin family, however, this interaction needs more than a single intracellular amino acid (33, 35). Lysine 225 was, however, also not mandatory for shedding, because lysine 225 is deleted in Tim-3Δ11 without affecting PMA-induced shedding. Using flow cytometry, we excluded the possibility that Tim-3Δ1CD is not shed by ADAM17, because of defective transport to the plasma membrane. We therefore conclude from these experiments that the intracellular domain is not needed to facilitate efficient ADAM10 and ADAM17 shedding of Tim-3, with the exception of the deletion of the complete intracellular domain. We hypothesize, that cell-surface localization or integration of Tim-3Δ1CD has changed in comparison to wild-type Tim-3. This might influence accessibility of Tim-3 or the cleavage site for ADAM17, respectively. It has been proposed that inhibition of L-selectin shedding by calmodulin is mediated by such a process, in which calmodulin binds to amino acids of the intracellular and transmembrane domain, thereby changing the conformation of the stalk region and reducing the accessibility for ADAM proteases (37). Whether such a mechanism might also contribute to Tim-3 shedding has to be investigated in future experiments.

Next, we showed that endogenously expressed Tim-3 is also shed by ADAM10 and ADAM17 after PMA or ionomycin stimulation, respectively. Tim-3 is expressed on human peripheral blood CD14+ monocytes (17). Moreover, LPS was recently shown to down-regulate Tim-3 from PBMCs by an unknown mechanism (17). Here, we demonstrate that mainly ADAM10 and to a lesser extend also ADAM17 are responsible for LPS-induced down-regulation of Tim-3, indicating that shedding of Tim-3 might contribute to the immune status of CD14+ monocytes. Our data did, however, not exclude the possibility that other ADAM- or MMP-proteases are involved in Tim-3 shedding. MMP-3 and MT1-MMP were shown to efficiently shed Tim-3 and to a lesser extend also ADAM17 are responsible for LPS-induced down-regulation of Tim-3, indicating that shedding of Tim-3 might contribute to the immune status of CD14+ monocytes. Our data did, however, not exclude the possibility that other ADAM- or MMP-proteases are involved in Tim-3 shedding. MMP-3 and MT1-MMP were shown to efficiently shed Tim-3 and to a lesser extent also ADAM17 are responsible for LPS-induced down-regulation of Tim-3, indicating that shedding of Tim-3 might contribute to the immune status of CD14+ monocytes.

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