Evaluation of the Contribution of Different ADAMs to Tumor Necrosis Factor α (TNFα) Shedding and of the Function of the TNFα Ectodomain in Ensuring Selective Stimulated Shedding by the TNFα Convertase (TACE/ADAM17)*

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Tumor necrosis factor-α (TNFα), a potent pro-inflammatory cytokine, is released from cells by proteolytic cleavage of a membrane-anchored precursor. The TNF-α converting enzyme (TACE; a disintegrin and metalloprotease17; ADAM17) is known to have a key role in the ectodomain shedding of TNFα in several cell types. However, because purified ADAMs 9, 10, and 19 can also cleave a peptide corresponding to the TNFα cleavage site in vitro, these enzymes are considered to be candidate TNFα sheddases as well. In this study we used cells lacking ADAMs 9, 10, 17 (TACE), or 19 to address the relative contribution of these ADAMs to TNFα shedding in cell-based assays. Our results corroborate that ADAM17, but not ADAM9, -10, or -19, is critical for phorbol ester- and pervanadate-stimulated release of TNFα in mouse embryonic fibroblasts. However, overexpression of ADAM19 increased the constitutive release of TNFα, whereas overexpression of ADAM9 or ADAM10 did not. This suggests that ADAM19 may contribute to TNFα shedding, especially in cells or tissues where it is highly expressed. Furthermore, we used mutagenesis of TNFα to explore which domains are important for its stimulated processing by ADAM17. We found that the cleavage site of TNFα is necessary and sufficient for cleavage by ADAM17. In addition, the ectodomain of TNFα makes an unexpected contribution to the selective cleavage of TNFα by ADAM17: it prevents one or more other enzymes from cleaving TNFα following PMA stimulation. Thus, selective stimulated processing of TNFα by ADAM17 in cells depends on the presence of an appropriate cleavage site as well as the inhibitory role of the TNF ectodomain toward other enzymes that can process this site.

TNFα is a pro-inflammatory cytokine that has a critical role in autoimmune disorders such as rheumatoid arthritis and Crohn’s disease (1, 2). TNFα is synthesized as a trimeric type II membrane-anchored precursor referred to as pro-TNFα (3). Upon cleavage in the juxtamembrane domain, the mature form of TNFα is released from the cell and can enter the blood stream (4, 5). This proteolytic release of TNFα from the membrane is referred to as “protein ectodomain shedding” (6, 7). Protein ectodomain shedding also affects the function of a variety of other structurally and functionally diverse molecules on the cell surface, including cytokines and growth factors, their receptors, adhesion proteins, and other molecules, such as the amyloid precursor protein, Notch and Delta (6–9).

Because of the critical role of TNFα in rheumatoid arthritis, considerable efforts have been made to identify the TNFα convertase. ADAM17 (a disintegrin and metalloprotease 17, also referred to as TNFα converting enzyme or TACE) is considered to be an important, if not the major, sheddase for TNFα (10, 11). ADAM17 was initially purified based on its ability to process a peptide, which mimics the physiological cleavage site of TNFα, in exactly the same position that is used by the TNFα converting activity in cells (see Table I and Refs. 10 and 11). A targeted deletion of ADAM17 in mice revealed a critical role in TNFα shedding in T cells (11). ADAM17 has also been shown to be the major sheddase of several other proteins, including transforming growth factor α (12, 13), heparin-binding epidermal growth factor-like growth factor (13–15), fractalkine (16), p75 neurotrophin receptor (17), and MUC1 (18). Besides ADAM17, additional candidate TNFα convertases have emerged from biochemical studies. ADAM10, which is most closely related to ADAM17, also can cleave a TNFα peptide at the physiological cleavage site in vitro (see Table I and Ref. 19). Furthermore, the TNFα cleavage site peptide can be processed by recombinant soluble forms of ADAMs 9 and 19 in vitro, although the cleavage sites for these ADAMs do not match the physiologically relevant site (see Table I and Refs. 20 and 21). In addition to these ADAMs, several other enzymes have been implicated in TNFα shedding. MMP7/matrilysin is critical for TNFα shedding in a mouse model for resorption of herniated discs (22), whereas the serine protease PR3 can release TNFα

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¶ The abbreviations used are: TNFα, tumor necrosis factor α; ADAM, a disintegrin and metalloprotease; TACE, TNFα converting enzyme; CHO, Chinese hamster ovary cells; mEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PV, pervanadate; wt, wild type; TRANCE, TNF-related-activation-induced cytokine.

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under conditions where it is highly expressed, such as in acute local inflammatory processes (23).

The ability of other ADAMs besides ADAM17 to cleave a TNFα peptide in vitro raises the question of whether these ADAMs may also contribute to TNFα shedding in vivo. To address this question, we evaluated TNFα shedding in cells isolated from wild-type, adam9−/−, adam10−/−, adam17−/−, and adam19−/− mice. In addition, we overexpressed different ADAMs to test which one(s) is(are) capable of processing pro-TNFα in cell-based assays. Our results confirmed that ADAM17 is the major stimulated sheddase of TNFα, at least in mouse embryonic cells. In addition, we found that ADAM19 is also able to cleave TNFα and may therefore contribute to TNFα shedding in cells. Finally, we addressed the requirements for cleavage of TNFα by ADAM17 through testing how mutations in TNFα affect its shedding by ADAM17. These studies revealed that an inhibitory function of the TNFα module toward other enzymes contributes to the selective role of ADAM17 in stimulated cleavage of TNFα.

MATERIALS AND METHODS

Reagents—All chemicals and reagents were purchased from Sigma unless otherwise indicated. Taconic metal fiber was harvested from BD Biosciences, concanavalin A-Sepharose and Protein G-Sepharose were purchased from Amersham Biosciences. BB94 was kindly provided by J. D. Becherer (GlaxoSmithKline, Research Triangle Park, NC).

Expression Vectors—The pAP-TNFα and pAP-TRN (TRANCE) expression constructs have been described previously (21, 24). In both constructs, an alkaline phosphatase moiety is attached to the C terminus of the full-length wild-type protein. To generate pAP-ΔTNFα, a PCR fragment encoding the amino acids 1–87 of TNFα was cloned into pAPltag5 vector (Genehunter Corp.) between the NheI and BglII sites. The resulting mutant form of TNFα lacks the ectodomain but contains the juxtamembrane domain, including the cleavage site for ADAM17. The pAP-TNF-Ecto (TRN) construct encodes a chimeric molecule in which the ectodomain of TNFα is replaced with that of TRANCE/OPGL. It was generated from previously described chimeras (25) by subcloning into the pAPltag5 vector. pAP-TNF-Ins (TRN) was generated by cloning PCR fragments encoding the cytoplasmic domain of TNFα (amino acid residues 1–53), the membrane-proximal juxtamembrane domain of TRANCE (amino acid residues 72–115), and the juxtamembrane domain and ectodomain of TNFα (amino acid residues 54–233) into pAPltag5. pAP-TNF-Ins (TRN) gives rise to a mutant form of TNFα with a portion of the TRANCE juxtamembrane domain inserted between the transmembrane domain and the juxtamembrane domain of TNFα. The pAP-ΔTNF-Ins (TRN) was generated by inserting PCR fragments encoding the cytoplasmic domain of TNFα (amino acid residues 1–53), the membrane-proximal juxtamembrane domain of TRANCE (amino acid residues 72–115), followed by the juxtamembrane domain of TNFα (amino acid residues 54–87) into pAPltag5. A diagram of the constructs used in this study is presented below in Fig. 3A.

Cell Culture, Transfection, and Ectodomain Shedding Assays—CHO cells were maintained in F-12 medium with 5% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. COS-7 cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 5% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin G, and 100 μg/ml streptomycin. Primary mouse embryonic fibroblasts (mEFs) lacking one or more ADAMs were isolated from corresponding adams−/− knockout mice as previously described (15, 21, 26, 27). mEFs and immortalized adams10−/− and adams19+/- cells (27) were grown in Dulbecco’s modified Eagle’s medium with 10% (v/v) fetal bovine serum. Cells seeded in six-well tissue culture plates (Falcon) were transfected with the appropriate expression plasmids using LipofectAMINE2000 (Invitrogen). The transfection solution was removed after 5 h, and cells were allowed to recover in complete medium overnight. To measure shedding of the introduced AP-fusion proteins under basal conditions, cells were washed once with PBS, and then cultured in Opti-Mem for 1 h. The Opti-Mem medium was collected and replaced with fresh Opti-Mem medium with 25 ng/ml phorbolester or 1 μg/ml batimastat and incubated for 1 h at 4 °C (for the hydroxamic acid-type metalloprotease inhibitor batimatstat (BB94) at the indicated concentration for another hour to assess shedding under stimulated or inhibited conditions. The change in ectodomain shedding upon addition of each activator or inhibitor of shedding was analyzed as described previously (15, 24). Finally, bafilomycin A, a specific inhibitor of vacuolar type H+/ATPase that prevents acidification of the lysosome, was added to cells to assess what role lysosomal protein degradation has in generating C-terminal stubs in cells expressing TNFα or ΔTNFα. After CHO cells were transfected with either TNFα or ΔTNFα and had recovered overnight, they were incubated with or without 10 μg/ml bafilomycin A1 for 1 h. Then the cells were lysed in cell lysis buffer, and the extracts were subjected to Western blot analysis as described below. All experiments were repeated at least three times with very similar results.

Western Blot and Immunoprecipitations—To prepare samples for Western blot analysis, transfected cells were washed with PBS, then lysed in 500 μl of cell lysis buffer (PBS, pH 7.4, with 1% (v/v) Triton X-100, 1 mM 1,10-phenanthroline) per well. Lysates were cleared by centrifugation at 13,000 × g for 30 min then incubated with concanavalin A-Sepharose for 2 h at 4 °C. After washing twice with cell lysis buffer and once with PBS, the beads were incubated with 2× sample loading buffer containing 10 mM diithiotreitol at 95 °C for 5 min. The samples were then separated by 15% SDS-PAGE, transferred to nitrocellulose, incubated with antibodies against ADAM9 (28), ADAM10 (CHEMICON International), or ADAM19 (21), and developed using a chemiluminescence detection system as described previously (28). For immunoprecipitations, cell lysates prepared as described above were cleared by centrifugation at 13,000 × g for 30 min and then incubated with anti-FLAG M2 monoclonal antibody (Sigma) overnight, followed by Protein G-Sepharose Fast Flow beads for 1 h at 4 °C. After washing twice with cell lysis buffer and once with PBS, the beads were incubated with 2× sample loading buffer containing 10 mM diithiotreitol at 95 °C for 5 min. The samples were then separated by 15% SDS-PAGE, transferred to nitrocellulose, and subjected to Western analysis with anti-FLAG M5 antibodies as described previously (28).

RESULTS

TNFα Shedding in Cells Lacking Candidate ADAM Sheddases—To address whether ADAMs 9, 10, or 19 make a significant or at least detectable contribution to the ectodomain shedding of TNFα in mouse embryonic fibroblasts, we compared TNFα shedding in cells derived from wild-type mice and mice lacking ADAMs 9, 10, 17, or 19 (12, 26, 27, 29). As shown in Fig. 1A, TNFα was shed constitutively in wild-type primary mouse embryonic fibroblasts mEFs, and its release could be stimulated by addition of either 25 ng/ml PMA or 100 μM pervanadate (PV). When constitutive and stimulated TNFα shedding was assayed in adams9−/−, adams10−/−, and adams19−/− cells, no difference compared with wild-type mEF was observed (Fig. 1A). Thus ADAMs 9, 10, and 19 are dispensable for the constitutive or stimulated shedding of TNFα in mouse embryonic cells, even though all three ADAMs are expressed in these cells (15, 26, 27, 29). On the other hand, PMA-stimulated TNFα shedding was abolished, and FV-stimulated shedding was strongly reduced in adams17−/− mEFs (Fig. 1B). Both PMA- and FV-stimulated shedding could be rescued by reintroduction of ADAM17 cDNA into adams17−/− mEFs (Fig. 1B). These findings confirm that ADAM17 has a critical role in PMA- and FV-stimulated shedding of TNFα in mEF cells. Interestingly, constitutive TNFα shedding was still observed in adams17−/− mEFs, although it was slightly increased after reintroduction of wild-type ADAM17 cDNA (Fig. 1B). This result suggests that ADAM17 as well as one or more other minor TNFα sheddases contribute to constitutive shedding of TNFα.

| ADAM | TNFα peptide cleavage site in vitro |
|------|----------------------------------|
| ADAM9 | SPLA-QA/VRSSRR |
| ADAM9 | SPLAQAVR-SSR |
| ADAM10 | SPLAQAVRSSSR |
| ADAM17 | SPLAQAVRSSSR |
| ADAM19 | SPLAQAVRSSSR |

TABLE I

Position of the in vitro cleavage sites for ADAMs 9, 10, 17, and 19 in a TNFα cleavage site peptide
Role of Different ADAMs in Shedding TNFα

Effects of Overexpression of ADAMs 9, 10, 17, and 19 on TNFα Shedding—Although ADAMs 9, 10, and 19 are not essential for constitutive or stimulated shedding of TNFα in mEF cells, one or more of these ADAMs could conceivably contribute to shedding of TNFα, especially in cells or tissues where they are highly expressed. To address this possibility, we tested whether overexpression of ADAMs 9, 10, 17, or 19 enhances constitutive or stimulated TNFα shedding in either CHO cells, COS-7 cells, or in adam10−/− or adam17−/− embryonic cells. When TNFα was co-expressed with wild-type ADAM9 in CHO or COS-7 cells, no change in its constitutive or stimulated shedding was observed in comparison to control experiments with the inactive mutant form ADAM9E>A (Fig. 2, A and B). When ADAM19 was co-expressed with TNFα in COS-7 or CHO cells, this led to an increased constitutive shedding of TNFα, whereas co-expression with the catalytically inactive ADAM19E>A did not (Fig. 2, A and B). Separate Western blot experiments confirmed that the wild-type and E>A mutant forms of ADAM9 as well as of ADAM19 were expressed at comparable levels in these experiments (data not shown). Interestingly, constitutive and stimulated shedding of TNFα was not noticeably increased when ADAM17 was co-expressed in CHO or COS-7 cells (Fig. 2, A and B).

Because the contribution of ADAM19 and other ADAMs to stimulated shedding may be obscured by the activity of ADAM17 in COS-7 and CHO cells, we also performed co-expression experiments of TNFα with different ADAMs in adam17−/− primary mEF and in immortalized adam17−/− mEF cells (referred to as E2 cells). Overexpression of ADAM19 in adam17−/− cells resulted in increased unstimulated shedding of TNFα, yet no additional increase was achieved following stimulation with PMA (Fig. 2C). This suggests that the catalytic activity of ADAM19 is not sensitive to PMA stimulation, which is consistent with a previous study in which ADAM19-dependent cleavage of TRANCE/OPGL was also not stimulated by PMA (29). As had been seen in CHO and COS-7 cells, overexpression of ADAM9 and ADAM9E>A also did not affect constitutive or stimulated shedding of TNFα in adam17−/− cells (Fig. 2D), even though ADAM9 and ADAM9E>A were expressed at similarly high levels (data not shown). In addition, we did not observe any noticeable increase in constitutive or stimulated shedding of TNFα when we overexpressed ADAM10 in adam17−/− cells (data not shown), which is consistent with the results of a previous study by Reddy et al. (30).

To further explore a potential role of ADAM10 in TNFα shedding, we repeated a similar experiment in adam10+/- and adam10−/− cells. Shedding of the EGF-receptor ligand betacellulin was used as a positive control for the catalytic activity of ADAM10 (15). The upper panel in Fig. 2E shows soluble forms of alkaline-phosphatase-tagged betacellulin in the supernatant of transiently transfected adam10+/- cells. The faster migrating form (marked by an arrow) is generated by ADAM10-dependent shedding; it is not detectable in the supernatant of adam10−/− cells, but is recovered when adam10−/− cells are rescued by co-transfection with wild-type ADAM10. The slower migrating form of betacellulin is presumably generated by an activity that is resistant to metalloprotease inhibitors (15). In a parallel experiment, co-expression of ADAM10 with TNFα in adam10−/− cells did not increase constitutive shedding of TNFα compared with adam10−/− cells expressing only TNFα (Fig. 2E, lower panel).
Role of Different ADAMs in Shedding TNFα

To learn more about how the ectodomain of TNFα may prevent other PMA- and PV-stimulatable enzymes from processing the TNFα cleavage site in cells, we evaluated the shedding of a mutant in which the ectodomain of TNFα was replaced by that of the TNF family member TRANCE (TNF-TRN). Shedding of this chimera could be stimulated by PMA in *adam17*−/− cells (Fig. 3F), suggesting that the ectodomain of a different TNF family member is not sufficient to prevent stimulated processing of the TNFα juxtamembrane domain in *adam17*−/− cells. In addition, PV-stimulated shedding of TNF-TRN in *adam17*−/− cells could be further enhanced by co-expression of ADAM17 (Fig. 3F).

The ADAM17 cleavage site in TNFα is in close proximity to the transmembrane domain (20 amino acid residues from the membrane). To test whether the cleavage site of TNFα must be in this membrane-proximal position to be processed by ADAM17, we inserted the juxtamembrane domain of TRANCE (amino acid residues 72–115) between the transmembrane domain and the cleavage site of both TNFα and ΔTNFα to create TNF-Ins(TRN) and ΔTNF-Ins(TRN) (Fig. 4A). Because the juxtamembrane domain of TRANCE contains two cysteine residues that most likely form a disulfide bond, this insertion presumably places a peptide loop between the cleavage site of TNFα and its transmembrane domain. In CHO and COS-7 cells, both mutants behaved similarly to wild-type TNFα in their shedding could be stimulated by PMA and PV (Fig. 4A). The released ectodomains of TNF-Ins(TRN) and ΔTNF-

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**Fig. 2. Evidence for a role of ADAM19, but not ADAMs 9 or 10 in shedding of TNFα.** A, effect of overexpressing different wild-type ADAMs as well as ADAMs carrying an inactivating E→A mutation in their catalytic site on constitutive and PMA-stimulated TNFα shedding in CHO cells. No evident difference in the PMA-stimulated shedding of TNFα was observed when ADAMs 9, 9E→A, 10, 17, 19, and 19 E→A were co-expressed. However, in the presence of co-expressed ADAM19, constitutive TNFα shedding was slightly, but detectably increased. This type of an increase in TNFα shedding was never seen when any of the other wild-type or mutant ADAMs analyzed here were co-expressed. B, upper panel: corroboration of the increased constitutive shedding of TNFα in the presence of overexpressed ADAM19 through a side-by-side comparison of constitutive shedding in the presence of co-expressed ADAM9, ADAM9E→A, ADAM10, ADAM17, ADAM17E→A, ADAM19, ADAM19E→A, or pcDNA3 as control. Constitutive shedding of TNFα was increased by overexpression of ADAM19, but not by overexpression of ADAM19E→A, or of other ADAMs or their catalytically inactive mutants. Lower panel: co-expression of ADAM19 or ADAM19E→A with TNFα in COS-7 cells further substantiates the result obtained in CHO cells in a different cell line. Overexpression of ADAM19 in COS-7 cells also increases the levels of a shed form of TNFα, which migrates slightly faster than TNFα shed by ADAM17. C, co-expression of TNFα with ADAM19 in immortalized *adam10*−/− mEF cells (E2) increases the amount of TNFα shed. D, constitutive shedding of TNFα compared with controls in which TNFα was co-expressed with ADAM19 E→A or with the empty vector (pcDNA3). The more pronounced effect of co-expression of ADAM19 on TNFα shedding in E2 cells compared with COS-7 cells and CHO cells is presumably due to the absence of ADAM17, which also participates in constitutive TNFα shedding (see Fig. 1B). D, TNFα was co-transfected with vector, ADAM9 or ADAM9E→A in *adam17*−/− E2 cells. Similarly high expression levels of ADAM9 and ADAM9E→A were confirmed separately by Western blot analysis (data not shown), and shedding of EGFP by overexpressed ADAM9 in parallel experiments was used to verify that ADAM9 is active in cell based assays (data not shown). No difference in constitutive or stimulated shedding of TNFα was observed in cells expressing ADAM9 versus the inactive ADAM9 E→A, shedding of betacellulin and TNFα in *adam10*−/− cells and in *adam10*−/− cells with or without co-transfected ADAM10. Upper panel: experiments with betacellulin, a known substrate of ADAM10 (15), are included as positive control for ADAM10 activity. The shed form of betacellulin generated by ADAM10 (marked by arrow) is present in *adam10*−/− cells, but not in *adam10*−/− cells. ADAM10-dependent shedding of betacellulin in *adam10*−/− cells can be rescued by co-transfection with ADAM10 (see also Ref. 15), confirming that ADAM10 is active in these experiments. The slower migrating shed form of betacellulin, which is the major form seen in *adam10*−/− cells, is generated by a separate activity that is not sensitive to BB94 (15). Lower panel: under identical conditions, no difference in constitutive or PMA-dependent shedding of TNFα was seen in *adam10*−/− cells in the presence or absence of reintroduced ADAM10.
The ectodomain of TNFα contributes to the selective shedding by ADAM17 in mEF cells by inhibiting one or more other activities that are capable of cleaving the TNFα juxtamembrane domain. A, schematic representation of TNFα, of TRANCE, and of the different chimeric constructs used in this study. CT, cytotail; TM, transmembrane domain; JM, juxtamembrane domain; ET, ectodomain; AP, alkaline phosphatase tag. A green triangle indicates the position of the FLAG tag. ΔTNFα, ectodomain deletion mutant of TNFα; TNF-TRN, TNFα in which the TNF module has been replaced with the TNF module of TRANCE/OPGL. TNFα-Ins(TRN), full-length TNFα with an insertion of the juxtamembrane domain of TRANCE (amino acids 72–115) between the transmembrane domain and juxtamembrane domain of TNFα; ΔTNFα-Ins(TRN), ectodomain deletion mutant of TNFα-Ins(TRN). All constructs used in these experiments have a FLAG tag at the N terminus and an AP tag followed by a Myc and His tag at the C terminus (the Myc and His tags are not shown on the diagram, see "Materials and Methods" for details). B, shedding of TNFα-AP compared with a mutant TNFα in which the ectodomain is deleted (ΔTNFα) in CHO cells. In ΔTNFα, the AP module is attached directly to the juxtamembrane domain of TNFα, which includes the cleavage site for ADAM17. In CHO cells, TNFα and ΔTNFα are shed constitutively, and shedding of both proteins can be stimulated by PMA and pervanadate (PV). C, shedding of TNFα and ΔTNFα in adam17−/− mEF cells. Shedding of full-length TNFα in adam17−/− mEF cells is not stimulated by PMA. PMA-stimulated shedding can be rescued by co-expression of ADAM17 (left panel, see also Fig. 1B). In contrast, PMA-stimulated shedding of ΔTNFα occurs in adam17−/− mEF cells that have not been rescued with wild-type ADAM17. This result suggests that the ectodomain of TNFα can inhibit PMA-dependent processing of TNFα by an activity or activities that are distinct from ADAM17. D, the PMA-induced shedding of ΔTNFα in adam17−/− cells can be further
brane stubs were generated in cells expressing \textit{H9004} and the supernatant as well as the expression levels of the full-\textit{adam17} PMA or PV in \textit{H9251} cells. Finally, co-expression of ADAM17 with \textit{adam17} even when the cleavage site is moved away from the plasma membrane. It should be noted that, because PMA- and PV-stimulated shedding of TNF \textit{adam17} – constructs, i.e. whether or not there is a clearly detectable increase in constitutive or stimulated shedding of TNF \textit{adam17} in a cell-based assay. In the case of \textit{adam9} and 10, we found no evidence for a contribution of these enzymes to constitutive or stimulated TNF \textit{adam17} release in co-expression experiments with TNF \textit{adam17}. Taken together with the results of loss of function experiments with \textit{adam9} and \textit{adam10} – cells, these findings argue against a role for \textit{adam9} and \textit{adam10} in TNF \textit{adam17} shedding in cells. Perhaps there are additional constraints on processing of the TNF \textit{adam17} cleavage site in a more physiological context, i.e. when membrane-anchored TNF \textit{adam17} is presented to a membrane-anchored ADAM in cells compared to an in \textit{vitro} situation, where a soluble peptide is cleaved by a soluble recombinant ADAM. Nevertheless, these results can not completely rule out the possibility that \textit{adam9} and \textit{adam10} may cleave TNF \textit{adam17} in other cells or tissues, for example in the presence of putative cofactors that might not be expressed in mEF cells.

When \textit{adam17} was co-expressed with TNF \textit{adam17} in CHO cells, there was a clear increase in constitutive, but not in stimulated TNF \textit{adam17} release. This is consistent with the results of a previous characterization of \textit{adam19}, in which it was found to be a constitutively active enzyme that was not significantly stimulated by PMA in cell-based assays (29). Some soluble TNF \textit{adam19} generated by \textit{adam19} migrates slightly faster than soluble TNF \textit{adam19} released by \textit{adam19}, indicating that \textit{adam19} cleaves TNF \textit{adam17} at a different site than \textit{adam19}. This finding can most likely be explained by the observation that \textit{adam17} and \textit{adam19} cleave the TNF \textit{adam17} cleavage site peptide in different positions in \textit{vitro} as well (see Table I). Taken together, these results demonstrate that \textit{adam19} can function as a constitutive TNF \textit{adam17} sheddase in cells. \textit{adam19} could thus conceivably make more significant contributions to constitutive TNF \textit{adam17} shedding in cells and tissues in which it is highly expressed, such as for example in the heart (29). Finally, overexpression of \textit{adam17} did not noticeably increase constitutive or stimulated shedding of TNF \textit{adam17} in CHO cells or COS-7 cells. Evidently the endogenous levels of \textit{adam17} are not a rate-limiting factor in TNF \textit{adam17} release. The lack of increase in constitutive shedding of

Role of Different ADAMs in Shedding TNF \textit{adam17}

Proteolytic release of TNF \textit{adam17} from its membrane-anchored precursor is thought to play an important role in regulating the physiological and pathological functions of this pro-inflammatory cytokine (3, 4, 10, 11, 31, 32). The TNF \textit{adam17} convertase (ADAM17) has been shown to be critical for processing TNF \textit{adam17} (10, 11), yet \textit{in vitro} studies have suggested that \textit{adam17} may also participate in the release of TNF \textit{adam17} from cells (19–21). Here we used mouse embryonic fibroblasts from \textit{adam9}–/–, \textit{adam10}–/–, and \textit{adam19}–/– mice to evaluate whether these three ADAMs might also participate in TNF \textit{adam17} shedding in \textit{vitro}. Even though \textit{adam9} and 10, and 19 are expressed in mEFs (15), no detectable defect in constitutive or stimulated shedding of TNF \textit{adam17} was found in \textit{adam9}–/–, \textit{adam10}–/–, or \textit{adam19}–/– mEFs. These results demonstrate that ADAM17 is the major FMA- and pervanadate-stimulated sheddase of TNF \textit{adam17} in mEF cells.

Even though “loss of function” experiments did not uncover any evidence for a role of \textit{adam9} and 10, or 19 in TNF \textit{adam17} shedding in mEF cells, this does not rule out that one or more of these ADAMs might contribute to TNF \textit{adam17} shedding, which might become more evident in cells or tissues where these ADAMs are highly expressed. However, to postulate that \textit{adam9}, \textit{adam10}, or 19 might participate in TNF \textit{adam17} shedding in \textit{vitro}, it is important to provide evidence that they are capable of cleaving TNF \textit{adam17} in a cell-based assay. In the case of \textit{adam9} and 10, we found no evidence for a contribution of these enzymes to constitutive or stimulated TNF \textit{adam17} release in co-expression experiments with TNF \textit{adam17}. Taken together with the results of loss of function experiments with \textit{adam9} and \textit{adam10} – cells, these findings argue against a role for \textit{adam9} and 10 in TNF \textit{adam17} shedding in cells. Perhaps there are additional constraints on processing of the TNF \textit{adam17} cleavage site in a more physiological context, i.e. when membrane-anchored TNF \textit{adam17} is presented to a membrane-anchored ADAM in cells compared to an in \textit{vitro} situation, where a soluble peptide is cleaved by a soluble recombinant ADAM. Nevertheless, these results can not completely rule out the possibility that \textit{adam9} and 10 may cleave TNF \textit{adam17} in other cells or tissues, for example in the presence of putative cofactors that might not be expressed in mEF cells.

When \textit{adam17} was co-expressed with TNF \textit{adam17} in CHO cells, there was a clear increase in constitutive, but not in stimulated TNF \textit{adam17} release. This is consistent with the results of a previous characterization of \textit{adam19}, in which it was found to be a constitutively active enzyme that was not significantly stimulated by PMA in cell-based assays (29). Some soluble TNF \textit{adam19} generated by \textit{adam19} migrates slightly faster than soluble TNF \textit{adam19} released by \textit{adam19}, indicating that \textit{adam19} cleaves TNF \textit{adam10} at a different site than \textit{adam19} and ADAM19. This finding can most likely be explained by the observation that \textit{adam17} and \textit{adam19} cleave the TNF \textit{adam17} cleavage site peptide in different positions in \textit{vitro} as well (see Table I). Taken together, these results demonstrate that \textit{adam19} can function as a constitutive TNF \textit{adam17} sheddase in cells. \textit{adam19} could thus conceivably make more significant contributions to constitutive TNF \textit{adam17} shedding in cells and tissues in which it is highly expressed, such as for example in the heart (29). Finally, overexpression of \textit{adam17} did not noticeably increase constitutive or stimulated shedding of TNF \textit{adam17} in CHO cells or COS-7 cells. Evidently the endogenous levels of \textit{adam17} are not a rate-limiting factor in TNF \textit{adam17} release. The lack of increase in constitutive shedding of enhanced by co-expressing wild-type \textit{adam17}. This confirms that \textit{adam17} can process the cleavage site of TNF \textit{adam17} in the absence of the TNF ectodomain. E, constitutive and stimulated shedding of TNF \textit{adam17} from \textit{adam17}–/– cells can be inhibited by 1 µM of the hydroxamic acid-type metalloprotease inhibitor batimatast (BB94), F, shedding profile of TNF \textit{adam17} (see panel A), which is similar to that of TNF \textit{adam17} in \textit{adam17}–/– cells. TNF-TRN shedding can be stimulated by PV, and stimulated shedding is further increased by co-expression ADAM17 WT in \textit{adam17}–/– cells. It should be noted that, because PMA- and PV-stimulated shedding of TNF \textit{adam17} is almost completely abolished in \textit{adam17}–/– cells, this study focuses on qualitative differences in the shedding profile of TNF \textit{adam17} versus TNF \textit{adam17}-constructs, i.e. whether or not there is a clearly detectable increase in shedding of these chimeric proteins after stimulation with PMA and PV. Nevertheless, semiquantitative information can be obtained by comparison of shedding levels for different samples within a given experiment. For example, the increase in stimulated shedding of constructs such as TNF \textit{adam17} and TNF-TRN from \textit{adam17}–/– cells after reintroduction of ADAM17 (see panels D and F, and Fig. 4E) was highly reproducible in any given experiment (n = 4), even though there was some variability in the strength of the overall effect of PMA or PV stimulation between experiments.
TNFα when ADAM17 is overexpressed in CHO cells also suggests that the activity of ADAM17 is tightly regulated, even when it is overexpressed.

What sequences in TNFα contribute to its selective cleavage by ADAM17? The structure/function analysis of TNFα revealed an unexpected role of the TNFα ectodomain in this process. Whereas stimulated shedding of TNFα is abrogated in

adam17−/− cells, shedding of ΔTNFα, which lacks the TNFα ectodomain, can be enhanced by PMA and PV in these cells. Evidently, the ectodomain of TNFα prevents one or more PMA- and PV-stimulated, BB-94-sensitive enzymes that are distinct from ADAM17 from cutting the TNFα cleavage site in cells. PMA-stimulated shedding of ΔTNFα was not affected in

adams9−/−, adam15−/−, or adam19−/− cells (data not

FIG. 4. Insertion of the juxtamembrane domain of TRANCE/OPGL between the transmembrane domain and cleavage site of TNFα does not affect selective processing by ADAM17. Shedding of chimeric TNFα molecules containing an inserted juxtamembrane domain of TRANCE/OPGL (see also the diagram in Fig. 3A) in CHO cells. Similar to TNFα and ΔTNFα (see Fig. 3B), shedding of both constructs was stimulated by PMA and pervanadate in CHO cells. B, Western blot of the lysates of CHO cells expressing TNFα (lane 1), ΔTNFα (lane 2), TNF-Ins(TRN) (lane 3), ΔTNF-Ins(TRN) (lane 4), or control (lane 5), probed with an antibody against the N-terminal (cytoplasmic) FLAG tag. The full-length proteins and membrane-anchored stubs are marked by arrows. The asterisk marks the C-terminal membrane stubs generated from TNFα-Ins(TRN) and ΔTNF-Ins(TRN), which are about 5 kDa larger than the C-terminal membrane stubs generated from TNFα and ΔTNFα. C, CHO cells transfected with TNFα or ΔTNFα were treated with 10 μg/ml bafilomycin A1 (an inhibitor of lysosomal acidification that blocks protein degradation in the lysosome). Bafilomycin A1 treatment resulted in increased levels of the TNFα C-terminal membrane stubs but had no evident effect on the levels of ΔTNFα C-terminal membrane stubs. D, the shedding profile of TNFα-Ins(TRN) resembles that of TNFα in adam17−/− mEF cells (see Figs. 1B and 3B); stimulated shedding of TNF-Ins(TRN) by PMA and PV is abrogated in

adams17−/− mEF cells but can be rescued by co-transfection with wild-type ADAM17. E, the shedding profile of ΔTNF-Ins(TRN) resembles that of ΔTNFα in adam17−/− mEF cells (see Fig. 3D) in that it can be stimulated by PMA. Co-transfection of ADAM17 further enhances the PMA dependent increase of ΔTNFα shedding.

adams15−/−, or adam19−/− cells (data not
shown), arguing against a major contribution of these ADAMs to cleaving ΔTNFα. Rescue of adam17−/− cells by transfection with wild-type ADAM17 further enhanced the PMA-stimulated ΔTNFα release compared with cells co-transfected with a control vector. This demonstrates that ADAM17 can recognize and process the membrane-anchored cleavage site of TNFα in cells even in the absence of the TNFα ectodomain. In this context it is interesting to note that PMA treatment also showed that deletions of eight or more amino acid residues in enzymes that are distinct from ADAM17. A previous study has demonstrated that a different TNF family ectodomain is not sufficient to cleaving ΔTNFα, arguing against a major contribution of these ADAMs to the TNFα cleavage site. Taken together, this study provides new insights into the ability of different candidate TNF family convertases to shed TNFα and into the unexpected contribution of the TNFα ectodomain in ensuring selective stimulated cleavage of TNFα by ADAM17.

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