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ADAM10 regulates FasL cell surface expression and modulates FasL-induced cytotoxicity and activation-induced cell death

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The apoptosis-inducing Fas ligand (FasL) is a type II transmembrane protein that is involved in the downregulation of immune reactions by activation-induced cell death (AICD) as well as in T cell-mediated cytotoxicity. Proteolytic cleavage leads to the generation of membrane-bound N-terminal fragments and a soluble FasL (sFasL) ectodomain. sFasL can be detected in the serum of patients with dysregulated inflammatory diseases and is discussed to affect Fas-FasL-mediated apoptosis. Using pharmacological approaches in 293T cells, *in vitro* cleavage assays as well as loss and gain of function studies in murine embryonic fibroblasts (MEFs), we demonstrate that the disintegrin and metalloprotease ADAM10 is critically involved in the shedding of FasL. In primary human T cells, FasL shedding is significantly reduced after inhibition of ADAM10. The resulting elevated FasL surface expression is associated with increased killing capacity and an increase of T cells undergoing AICD. Overall, our findings suggest that ADAM10 represents an important molecular modulator of FasL-mediated cell death.
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The Fas ligand (FasL, CD95L, Apo-1L, CD178) is a 40 kDa protein that belongs to the tumor necrosis factor (TNF) family of membrane-associated cytokines.[1] It can trigger apoptotic cell death through its default receptor Fas (see[2] for review). FasL is inducibly expressed in lymphocytes, particularly T cells, where it regulates immune-response termination by activation-induced cell death (AICD). Analyses of mice lacking Fas or FasL have indicated that FasL controls autoimmunity (see[3] for review). In addition, it is one of the major effector molecules of cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells involved in removing virally infected cells or cancer cells.[4,5]

In several cell types, a soluble form of FasL (sFasL) can be generated through metalloprotease (MPs)-mediated cleavage of the transmembrane molecule shortly after cell surface expression.[6–9] It has been observed that soluble FasL (sFasL) can have proapoptotic as well as antiapoptotic functions depending on the local microenvironment.[10,11] For both membrane-bound and sFasL, a spontaneous trimer formation has been suggested, which seems indispensable for triggering the death signal via Fas oligomers.[12] sFasL was found in sera of patients with large granular lymphocytic leukemia and NK cell lymphoma.[13] A number of more recent reports point to a correlation of sFasL serum levels with disease progression, for example, during the asymptomatic stage of HIV infection, in rheumatoid arthritis and osteoarthritis, in ulcerative colitis, and a large number of malignancies.[14]

There is, however, only limited knowledge about the proteolytic release of FasL from the membrane and the functional consequence of this proteolysis. Depending on the availability and accessibility of respective cleavage sites, matrix metalloproteases (MMPs) like MMP-7 have been proposed as FasL sheddases in different cellular systems mostly using *in vitro* proteolysis assays.[15,16] On the basis of observation that MMP-inhibiting tissue inhibitors of metalloproteases (TIMPs) did not alter FasL shedding, it was suggested that members of the 'a disintegrin and metalloprotease' (ADAM) family are responsible for the generation of sFasL.[6] This family of zinc-dependent transmembrane proteases has been implicated in the ectodomain shedding of various membrane-bound proteins.[17] ADAMs are generated as inactive zymogenes. During the maturation process in the late golgi compartment the prodomain is removed from the ADAM precursor protein, leading to the generation of the mature active protease. ADAMs play an important role in diverse biological processes such as fertilization, myogenesis, neurogenesis and the activation of growth factors and immune regulators such as TNF-α.[18] The TNF-α convertase ADAM17 was the first member of this family for which a role in ectodomain shedding has been found. It has been implicated

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Keywords: FasL; cell death; shedding
Abbreviations: ADAM, a disintegrin and metalloprotease; AICD, activation-induced cell death; CP-FasL, cell pellet FasL; FasL, Fas Ligand; MCD, methyl-β-cyclodextrin; MEFs, mouse embryonic fibroblasts; MMP, matrix metalloprotease; MP, metalloprotease; NTF, N-terminal fragment; PI, propidium iodide; SEA, staphylococcal enterotoxin A; TIMP, tissue inhibitor of metalloproteases
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in the proteolysis of several substrates like TNF-α, TNF-α receptors I and II, TGF-α, IL-6 receptor, and fractalkine.[19] The close relative ADAM10 has been shown to play important roles in development and in the central nervous system by influencing Notch/Delta activation and N-cadherin-dependent β-catenin signalling.[20,21] ADAM10-deficient mice die at day 9.5 of embryogenesis with multiple defects of the developing central nervous system, somites, and cardiovascular system.[20]

In the present study, we focused on the potential role of different ADAMs in the regulation of FasL-mediated functions. We demonstrate that ADAM10 is the major protease responsible for FasL ectodomain cleavage in murine fibroblasts and human T cells. We also show that ADAM10 activity regulates FasL surface expression and modulates FasL/Fas-dependent apoptosis and AICD.

Results

Inhibitor studies suggest an involvement of ADAM10 in FasL shedding. Human FasL (hFasL) is a type II transmembrane molecule with 281 amino acids (Figure 1a). It can be detected as a membrane-bound form with an approximate molecular weight of 40 kDa and as a soluble form of 26–29 kDa.

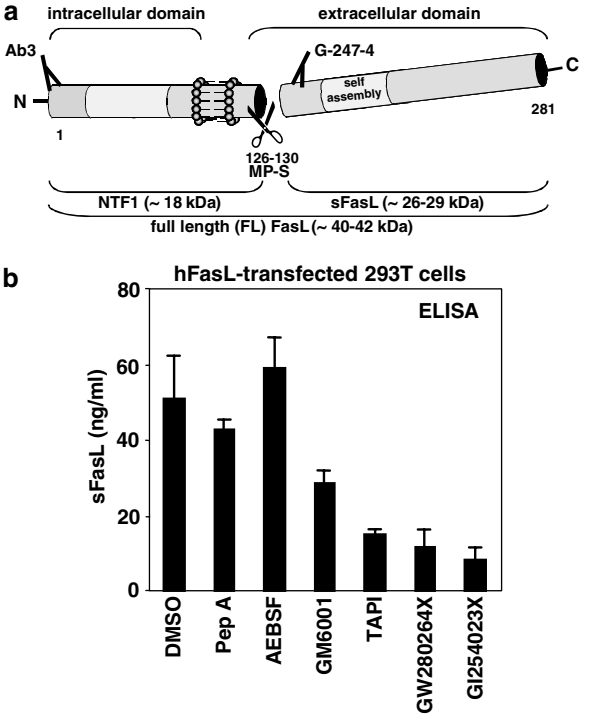

Figure 1 Effect of protease inhibitors on FasL cleavage in 293T cells. (**a**) Schematic representation of FasL. FasL is a type II transmembrane molecule with putative MP cleavage sites within the extracellular part (MP-S). The full-length (FL) 40 kDa FasL is cleaved in the extracellular domain by a protease activity, generating a soluble ectodomain (sFasL) and a membrane-bound 18 kDa NTF termed NTF1, which can be further processed. The antibody G247-4 reacts with an extracellular epitope, whereas Ab3 detects intracellular determinants. (**b**) 293T cells were transfected with hFasL. 6 h after transfection, cells were treated for 12 h with DMSO or the following protease inhibitors: pepstatin A (1 μM), AEBSF (100 μM), TAPI (50 μM), GM6001 (10 μM), GW280264X (5 μM), or GI254023X (5 μM). Supernatants were subjected to ultracentrifugation before ELISA detection of sFasL. One representative out of three experiments is shown

A putative protease ectodomain cleavage site of hFasL has initially been mapped to Lys129-Gln130 in murine cells[8] and to Ser126 and Leu127 in human 293 T cells.[11] These cleavage sites are located outside the region that is required for trimerization and self-assembly allowing the sFasL to form trimers. In agreement with this, the FasL mutant D4, which carries a deletion between aa 111 and 133, was shown to be uncleavable in murine cells.[8] We also found diminished release of sFasL in murine embryonic fibroblasts (MEFs) and in 293T cells expressing this mutant indicating that in both cell types the protein is cleaved in a similar region (Supplementary Figure 1).

To characterize the protease responsible for FasL cleavage, we studied the release of sFasL into the culture medium of FasL-transfected 293T cells by enzyme-linked immunosorbent assay (ELISA) using a panel of different inhibitors. It has been demonstrated that FasL-mediated apoptosis can also be owing to the secretion of FasL-bearing exosomes/microvesicles.[22] To exclude the detection of FasL released in exosomes into the culture medium, cell supernatants were subjected to ultracentrifugation before analyses. Although inhibitors of aspartyl and serine proteases (pepstatin A and AEBSF, respectively) did not show an obvious effect on FasL shedding, the wide-spectrum MP inhibitors GM6001 and TNF-α protease inhibitor (TAPI) led to a strong reduction of sFasL (Figure 1b). We also used two recently described hydroxamate-based compounds that differ in their capacity to block the activities of the two disintegrin-like MPs ADAM17 and ADAM10.[23] Whereas GW280264X potently blocks both enzymes, GI254023X possesses comparable inhibitory potency for ADAM10 only and blocks ADAM17 with more than 100-fold reduced potency. The preferential ADAM10 inhibitor GI254023X blocked the constitutive release of sFasL into the medium with a similar potency as the mixed ADAM10/ADAM17 inhibitor GW280264X (Figure 1b). The inhibitory effect of the latter two compounds was even more pronounced than the inhibition achieved by the two wide-spectrum MP inhibitors. These data suggest that ADAM10 may be responsible for the cleavage of FasL.

To explore further the involvement of ADAM10 in sFasL release, we used a cell-free *in vitro* cleavage assay. Recombinant ADAM10 was able to cleave recombinant FasL *in vitro* resulting in the generation of one N-terminal cleavage product after 5 h and an additional FasL fragment after 14 h with an apparent molecular weight of approximately 14 and 12 kDa, respectively (Figure 2). Incubation of FasL alone in reaction buffer or incubation with recombinant ADAM17 did not affect FasL proteolysis. This experiment confirms that ADAM10 is capable of generating FasL fragments and also suggests that ADAM10 might use two distinct FasL cleavage sites.

Loss of function and reconstitution experiments provide evidence for the specific role of ADAM10 in constitutive FasL shedding. To define more precisely the role of ADAMs in FasL shedding, we used a panel of established MEF cell lines that were generated from mouse embryos with a targeted deletion of different ADAM proteases as

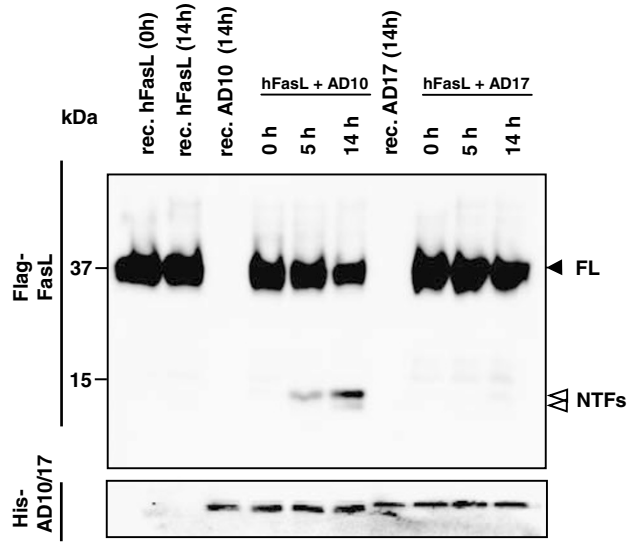

Figure 2 Recombinant human FasL is cleaved by recombinant human ADAM10 *in vitro*. Recombinant FasL was incubated with recombinant ADAM10 or ADAM17 in reaction buffer. Before starting the reaction, a control sample was taken, representing the intact FasL. After 5 h and after 14 h, samples were immediately mixed with laemmli buffer and analyzed on SDS-PAGE. ADAM10 and ADAM17 were detected by immunoblot with anti-His-antibodies. For FasL detection anti-Flag-antibodies against the N-terminal Flag tag were used. The blot is representative of three independent experiments. NTF: NTFs of FasL; FL: full-length FasL

described previously.[24] Whereas release of sFasL was not diminished in ADAM17$^{-/-}$, ADAM9$^{-/-}$, and ADAM15$^{-/-}$ MEFs compared to wild-type cells, the generation of sFasL was almost completely abolished in ADAM10-deficient MEFs (Figure 3a). The reduced capacity to generate sFasL was confirmed by immunoblot in two independent ADAM10-deficient cell lines (Figure 3b). The observed heterogeneity in size of full-length FasL has been described before and is most likely owing because of differential glycosylation.[25,26] Retransfection of ADAM10 into ADAM10$^{-/-}$ MEFs restored the generation of the sFasL (Figure 3c) as well as the appearance of membrane-bound N-terminal FasL fragments (NTFs) (Figure 3d). In contrast, ADAM17 transfection into ADAM10-deficient cells (Figure 3c and d) did not rescue the defective sFasL generation, highlighting the important role of ADAM10 in FasL shedding.

ADAM10 regulates endogenous FasL expression and localization in T cells. Next we analyzed whether ADAM10 is also involved in the constitutive or stimulated cleavage of endogenous FasL in human T lymphocytes. Therefore, human PBMCs were isolated from buffy coat preparations, stimulated with phytohaemagglutinin (PHA) and propagated in the presence of Interleukin-2 (IL2). The cells were restimulated with Con A in the presence or absence of drugs known to affect the shedding mediated by MPs. The expression of ADAM10 in these cells was confirmed by immunoblot analyses (Supplementary Figure 2). Whereas the calcium ionophor ionomycin and the cholesterol depleting agent methyl-β-cyclodextrin (MCD) only slightly increased the rate of sFasL generation as determined by ELISA, the MP inhibitors led to a striking reduction of sFasL release (Figure 4). The inhibitory effect was most pronounced using the preferential ADAM10 inhibitor GI254023X and the ADAM10/17 inhibitor GW280264X suggesting that the majority of endogenous FasL shedding is mediated by ADAM10.

Recently, we were able to demonstrate for *in vitro* propagated T cell clones that an initial upregulation of surface FasL is owing to the actin-dependent transport of FasL containing secretory vesicles to the cell surface. A second maximum of FasL expression starting approximately 60 min after induction of expression is owing to increased transcriptional expression of FasL leading to *de novo* protein synthesis.[27,28] To follow the role of ADAM10 in more detail, we analyzed the effect of the ADAM10 inhibitor GI254023X and the mixed ADAM10/ADAM17 inhibitor GW280264X in this assay system.[27] FasL surface expression of an established CD4$^+$ T cell clone was monitored over 180 min after induction of FasL expression with PMA/ionomycin (Figure 5a). The membrane-bound FasL expression followed the typical biphasic kinetic as described before.[27] Using the preferential ADAM10 inhibitor, we observed a strongly increased FasL-surface expression both at the phase of degranulation (20–40 min) and *de novo* synthesis (120 min). By microscopical inspection as well as by determination of scatter properties of the cells by flow cytometry, it became apparent that with increasing amount of surface-bound FasL more apoptotic T cells appeared (see below). Similar results were obtained in three separate experiments. In all instances, the ADAM10 inhibitor stabilized the surface expression of FasL during the observation period of up to 180 min.

The fluorescence activated cell sorting (FACS) data were supported by biochemical analyses showing that the expression of full-length FasL (intracellular and membrane-bound) follows the same biphasic kinetic. Stimulation of the CD4$^+$ T cell clone led to an increased level of full-length FasL after treatment with the ADAM10 inhibitor between 20 and 120 min of incubation (Figure 5b, upper panel). This increase was concomitant with a strong decrease in the ability to generate the intracellular NTF1 indicative of a preceding ectodomain shedding (Figure 5b, lower panel). ELISA analyses confirmed that GI254023X treatment also strongly affected the ability of primary T cells to generate sFasL in a time–dependent manner (Figure 5c). To strengthen our inhibitor studies, we performed additional experiments using siRNA-mediated downregulation of ADAM10 in T cells. The decrease of endogenous ADAM10 (insert, Figure 5d) correlated with a decrease in the release of sFasL as determined by ELISA (Figure 5d). Taken together, these data strongly suggest a pivotal function of ADAM10 in controlling the level of surface-bound and released FasL and thereby potentially affecting the survival rates in T cells.

ADAM10 modulates FasL-mediated cytotoxicity and activation-induced T cell death. To analyze whether ADAM10 inhibition would also affect the cytotoxic capacity of membrane-bound FasL, we preincubated stably FasL-transfected KFL-9 cells in the presence of GI254023X, GW280264X, TAPI, or dimethyl sulfoxide (DMSO) and analyzed the induced lysis of chromium-labelled Jurkat

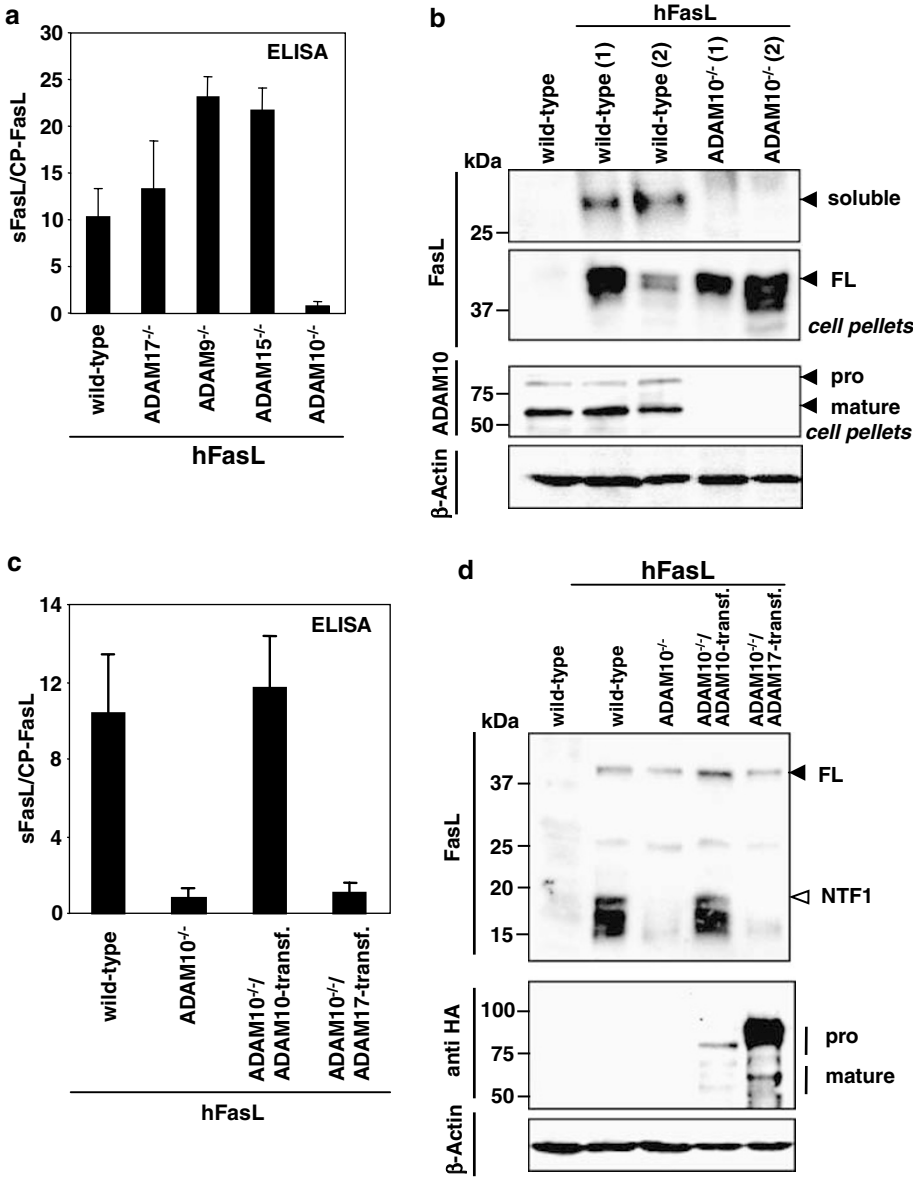

Figure 3 FasL cleavage is mediated by ADAM10 in fibroblasts. MEFs were transfected with hFasL. After 6 h, the culture medium was removed and the cells were cultured for 24 h before cells and supernatants were analyzed by immunoblot. For ELISA-analysis, sFasL generation over 12 h was measured. (**a**) Supernatants and pellets of the indicated MEFs were subjected to ELISA for detection of FasL. To normalize variations in transfection efficiency for each cell line sFasL generation was calculated as ratio of sFasL in the supernatant to FasL in cell pellets (CP-FasL). Data of three independent experiments are expressed as means ± standard error of the mean (S.E.M.). (**b**) Release of sFasL in the medium of untransfected and FasL-transfected wild-type MEFs and ADAM10$^{-/-}$ cells was analyzed by western blotting. Despite comparable amounts of full-length FasL (middle panel), sFasL was only detected in supernatants of FasL-transfected wild-type MEFs (upper panel). The expression of ADAM10 in the same cell lysates is shown in the lower panel. (**c**) FasL-transfected ADAM10$^{-/-}$ MEFs were retransfected with ADAM10 (ADAM10$^{-/-}$/ADAM10-transfected) or ADAM17 (ADAM10$^{-/-}$/ADAM17-transfected) and compared with wild type and ADAM10$^{-/-}$ MEFs for the production of sFasL by ELISA. Data of three independent experiments are expressed as means ± standard error of the mean (S.E.M.). (**d**) ADAM10$^{-/-}$ MEFs were retransfected with ADAM10 (ADAM10$^{-/-}$/ADAM10-transfected) and compared to wild type and ADAM10$^{-/-}$ and ADAM10$^{-/-}$/ADAM17-transfected cells for the generation of the FasL NTFs. Western blots are shown for FasL (Ab3), HA-tagged ADAM10, HA-tagged ADAM17 and β-actin. NTF, NTFs of FasL; FL, full-length FasL

cells (6a). All applied inhibitors increased FasL cell surface expression of the KFL-9 cells as determined by FACS analysis (data not shown). This FasL accumulation correlated with increased Jurkat cell death as determined by the chromium-release assay (Figure 6a). Killing was shown to be FasL-specific by blocking with Fas-Fc (data not shown), which is described as a potent inhibitor of FasL-induced cell death.[29] Thus, these data show that ADAM10-mediated cleavage also represents an important mechanism for regulating FasL-induced cytotoxicity.

Finally, to analyze if ADAM10 also contributes to the immunoregulatory phenomenon of AICD by regulating FasL surface expression, we used a previously described system of superantigen-stimulation. We stimulated cloned CD4$^+$ T cells

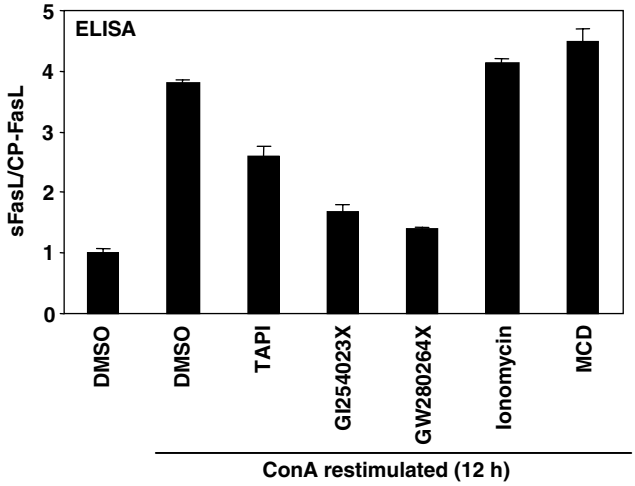

Figure 4 ADAM10 inhibition leads to reduced endogenous sFasL release in T cells. PHA-activated PBMCs were preincubated for 1 h in DMSO, TAPI (50 μM), GI254023 (5 μM), GW280264 (5 μM), ionomycin (20 ng/ml), and MCD (10 mM). The cells were restimulated by addition of 5 μg/ml Con A and sFasL released over 12 h was determined by ELISA. To normalize variations in FasL expression shedding was calculated as ratio of sFasL in relation to CP-FasL. One representative out of three experiments is shown. S.D. were calculated from triplicates

with Staphylococcus enterotoxin A (SEA).[30] Stimulation of SEA-reactive but SEE-insensitive clones with low dose of superantigen alone led to a twofold increase in FasL cell surface expression measured after 18 h and to a concomitant two- to three fold decrease in the number of viable T cells (Figure 6b and c). Treating the cells with superantigen in the presence of the ADAM10/ADAM17 inhibitor GW280264X caused an increase in FasL expression and also a further reduction of viable cells as judged by scatter properties and propidium iodide (PI)-staining. Again, the ADAM10 inhibitor GI254023X showed the most pronounced effect leading to the strongest stabilization of FasL surface expression and reduction in the number of viable cells (Figure 6c). Similar AICD experiments were performed six times with two different T cells clones at different time points after restimulation. Although the increase in FasL expression and the susceptibility to AICD varied dependent on the culture period after restimulation, in all experiments, the effect of ADAM10 inhibition became apparent. To support further the significance of this finding, we also performed four experiments with different clones in triplicates (two examples are shown in Supplementary Figure 4). FasL surface expression was significantly increased when cells were treated with GI254023X, whereas the number of viable cells significantly decreased compared to solvent-treated cells. In conclusion, ADAM10 inhibition led to a significant increase in FasL expression associated with a direct effect on cell survival and AICD.

Discussion

FasL plays a pivotal role in the regulation of apoptosis within and outside the immune system. It serves as a death factor during immune response termination by AICD[31] and for the establishment of immune privileged tissues.[32] Genetic defects of Fas/FasL-associated apoptosis pathways lead to autoimmune lymphoproliferative syndromes in mice and humans.[33] Moreover, tumors of different cellular origins might utilize FasL to escape the immune surveillance and counterattack Fas-expressing T cells.

Over the past years, it became evident that FasL might also be released by protease cleavage to act as a soluble cytokine.[8] However, the functional properties of the sFasL and the NTFs are still not well understood. Moreover, it is not yet clear which proteases are responsible for the release of the sFasL in T cells. Previous studies have suggested a MP to be responsible for the ectodomain shedding of FasL in other cell types.[7,8] MP inhibitors decreased the soluble and increased the membrane-bound form of FasL.[34] Several MMPs including MMP-3 (stromelysin-1)[35] and MMP-7 (matrilysin)[9,16] have been associated with FasL cleavage in different cellular systems. On the other hand, several other members of the TNF-family and TNF receptor family are proteolytically processed by ADAMs, suggesting that these proteases might also be involved in the FasL cleavage. However, a recent report excluded the TNF-α convertase ADAM17 as a possible FasL secretase,[36] leaving the potential role of other ADAM family members open.

In the present study, we have provided multiple lines of evidence that the MP ADAM10 is responsible for the initial and crucial proteolytic processing of FasL, leading to the generation of membrane-bound NTFs and a C-terminal soluble fragment which is released into the medium of cultured cells. Apart from the identification of ADAM10 as the major protease responsible for FasL cleavage in 293T cells, in MEFs and in primary and in established human T cells, we demonstrate how this cleavage is involved in the regulation of AICD and FasL-induced cytotoxicity.

In our experiments using 293T cells, we confirmed the importance of the juxtamembrane FasL cleavage. Initial evidence that ADAM10 is responsible for the release of sFasL was obtained in inhibition studies using broad spectrum MP inhibitors and inhibitors that we have previously shown to inhibit the activities of ADAM10 and ADAM17.

The assumption that ADAM10 is the major FasL sheddase was strongly supported by expression experiments in wild type and ADAM10-deficient MEFs. ADAM10 re-expression experiments rescued the ability to generate sFasL and membrane-bound NTFs, confirming the important role of this protease. Although, in principle, these effects could also be indirect, *in vitro* cleavage assay further underlined that ADAM10 is able to interact directly with FasL and to cleave this protein. Additionally, the sizes of the *in vitro* generated cleavage fragments are similar to the fragments generated in cell culture making a direct interaction of ADAM10 and FasL likely. Even though this assay does not allow any conclusion about the FasL processing kinetics *in vivo*, our analyses of *in vitro* propagated T cell clones showed that only 40 minutes after an initial FasL upregulation the full-length FasL disappears (Figure 5b). The same holds true for the second maximum of FasL expression after 60 min. As both effects are abrogated in the presence of the ADAM10 inhibitor GI254023X, it is likely that ADAM10

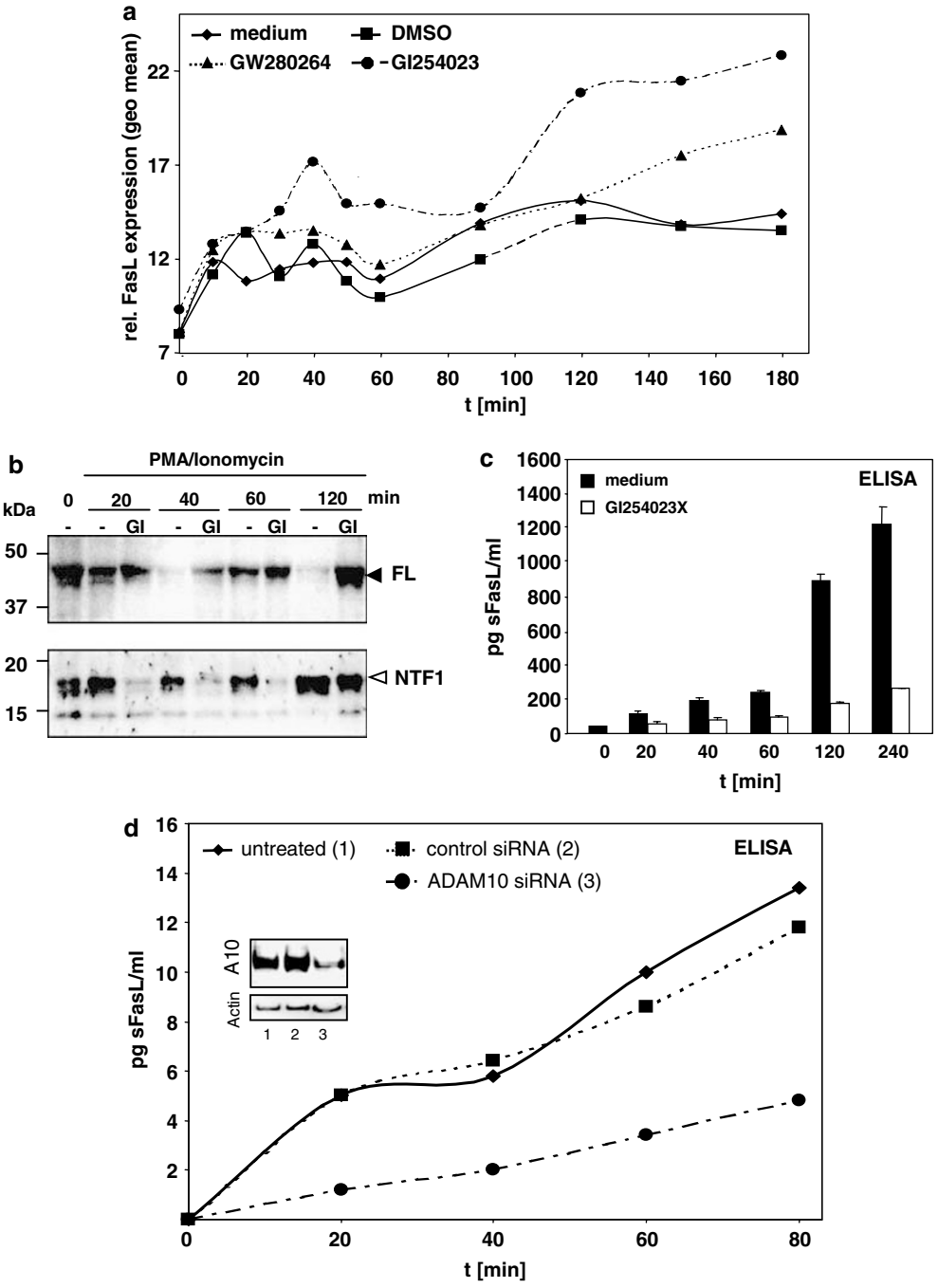

Figure 5 ADAM10-dependent FasL surface expression (**a**) FasL surface expression in cloned $CD4^+$ T cells was induced with PMA (10 ng/ml) and ionomycin (500 ng/ml) in the presence or absence of ADAM10 (GI254023) and ADAM10/17 (GW280264) inhibitors and monitored for 180 min using FACS analysis. One representative experiment of three is shown. (**b**) Western blot analysis of lysates of cloned $CD4^+$ T cells in a similar experimental setup using the N-terminal anti-FasL antibody. NTF, NTFs of FasL; FL, full-length FasL. (**c**) ELISA analysis of the supernatants of the induced T cells demonstrate that GI254023X treatment (open bars) led to a strong reduction in the ability to generate proteolytically sFasL as compared to untreated cells (filled bars). S.D. were calculated from triplicates. (**d**) The medium of untreated, mock-transfected or ADAM10 siRNA-transfected T cells (PHA blasts) was changed 72 h after transfection. Cells were stimulated with PMA/ionomycin and further incubated and supernatants were harvested at different time points and analyzed for sFasL release by ELISA. Cell pellets were analyzed for ADAM10 expression by immunoblot (insert)

is able to cleave membrane-bound FasL much faster *in vivo* than *in vitro*.

It has been reported previously that human FasL is cleaved in murine cells in between Lys129-Gln130,[8] whereas the cleavage occurs between Ser126 and Leu127 in human 293 T cells.[11] The different cleavage sites in mouse and human cells might be owing to cell type specific effects (e.g. glycosylation), which influence the fine specificity of FasL cleavage. They are also likely due to slight structural differences within the juxtamembrane region of human and murine FasL. Even

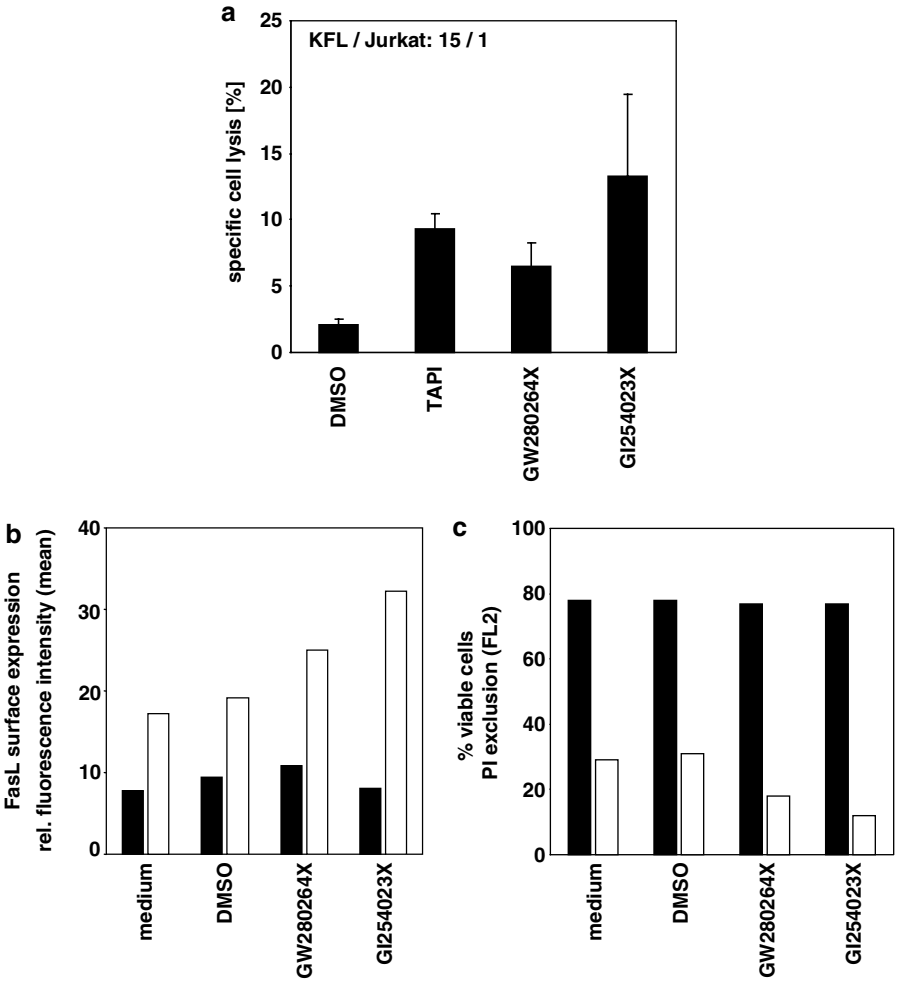

Figure 6 ADAM10 activity affects FasL-mediated cytotoxicity and activation-induced T cell death. (**a**) Jurkat target cells were labeled with ^{51}Cr and mixed with FasL-expressing KFL cells in the presence of DMSO, TAPI (50 μM), GI254023 (5 μM) or GW280264 (5 μM) at a ratio of 15:1. The cytotoxicity caused by FasL was measured by ^{51}Cr release after 20 h of co-incubation. S.D. were calculated from triplicates. (**b** and **c**) Increased AICD in T cells after inhibition of ADAM10. $CD4^+$ T cells were stimulated in the presence or absence of TAPI, GW280264X or GI254023X with SEA superantigen for 18 h (white bars) or left unstimulated (black bars). One representative out of six experiments is shown. (**b**) FasL surface expression was analyzed by flow cytometry using a PE-conjugated anti-FasL antibody. Relative surface expression is represented by mean values of the detected fluorescence intensity. (**c**) Cell viability was analyzed by flow cytometry using PI-staining of clone cells in the presence or absence of the indicated inhibitors

though human and mouse ADAM10 show 96% amino acid sequence homology, slight post-translational differences in the enzymes might explain the apparent heterogeneity in cleavage specificity. It should be noted that ADAM proteases do not generally use a very specific consensus site for substrate cleavage. Our analyses of an uncleavable human FasL mutant (Supplementary Figure 1) showed that the release of sFasL was completely abrogated in human and murine cells, indicating that despite the loose cleavage specificity the protease susceptible region is located within this region.

ADAM10 has been implicated before in the shedding of different substrates including CD44,[37] amphiregulin,[38] N-cadherin,[21] E-cadherin[24] and the neuronal adhesion molecule L1.[39] For some of these substrates, ADAM10 is responsible for both the constitutive and the inducible shedding. For other substrates, ADAM17 appears to mediate predominantly inducible shedding events. As judged from our immunoblot and ELISA data, the basic level of ADAM10-mediated FasL shedding is already very high in the analyzed cell lines. Therefore, it is not surprising that stimulation with well-known inducers of ADAM shedding such as MCD or ionomycin did not lead to a significantly increased level of sFasL release. A role for ADAM10 in FasL shedding did not only become evident in the experiments with heterologous expression in 293T cells or MEFs but is also reflected in experiments with untransformed human T cells. ADAM10 and FasL are coexpressed in these cells (Figure 5d and Supplementary Figure 2), indicating a functional link of both proteins. Using the preferential ADAM10 inhibitor GI254023X and siRNA-mediated downregulation of ADAM10, we could show that ADAM10 mediates the release of sFasL in T cells.

Our T cell immunoblot analyses showed that FasL processing is associated with the generation of two predominant N-terminal cleavage products, which could be reduced by the incubation with the preferential ADAM10 inhibitor. As ADAMs

do not use a specific consensus motif for substrate cleavage, these fragments might be due to distinct ADAM10 cleavage sites. This is in accordance with our *in vitro* cleavage assay, which indicates that ADAM10 is able to generate two FasL cleavage products. However, we cannot exclude that other proteases also contribute to the further degradation of the ADAM10-generated fragment(s). These fragments might even represent an identical protein fragment with different post-translational modifications.[40]

As FasL also acts as a signal transducing receptor mediating 'reverse signalling' in T cells,[40] it is tempting to speculate that the ADAM10-mediated processing might represent a prerequisite for further degradation, which could influence FasL-mediated signal transduction.

Moreover, FasL shedding through ADAM10 might serve as a mechanism to adjust the killing activity of T cells. In general, it is accepted that the membrane-bound FasL is the most effective activator of Fas *in vivo*. Therefore, the ADAM10-mediated shedding of FasL may represent an important regulatory mechanism for controlling the level of membrane-bound FasL. Indeed, our data indicate that ADAM10-mediated shedding can counteract the cytotoxic activity of the full-length FasL form. This assumption is supported by our findings that AICD in T cells is significantly increased after inhibition of ADAM10. This inhibition of ADAM10 activity causes a time–dependent increase of FasL surface expression and correlates with increased cell death. Dysregulation of ADAM10-mediated FasL cleavage may lead to insufficient FasL-induced apoptosis of T cells and could thereby contribute to chronic inflammation.

Various tumors have been described to express constitutively relatively high levels of membrane-bound FasL suggesting that this represents a mechanism to ensure tumor survival by blocking immune defense.[41] It remains to be investigated to which extent ADAM10 contributes to this process. In any case, the modulation of FasL shedding, FasL-induced killing or sFasL-induced masking of Fas molecules on tumor cells may provide an interesting therapeutical approach for different kinds of tumors.

In conclusion, we have shown that the MP ADAM10 is critically involved in the shedding of the FasL. This proteolysis may influence FasL-mediated cytotoxicity under physiological and pathophysiological conditions. The coordinated interaction of ADAM10 and FasL may therefore be of critical importance for the tight regulation of induced cell death in health and disease.

Materials and Methods

Primary antibodies, reagents and plasmids. For western blot detection of FasL, the C-terminal monoclonal antibody G247-4 (BD Pharmingen, San Diego, CA, USA) and the N-terminal polyclonal antibody Ab3 (Calbiochem, Bad Soden, Germany) were used. For flow cytometry, we used a PE-coupled monoclonal anti-FasL antibody (NOK-1, Caltag, Burlingame, CA, USA). ADAM10 was detected using the polyclonal antiserum B42.1 described previously[20] or an anti-HA-antibody (Roche, Mannheim, Germany). Anti-His-antibodies were purchased from Dianova, Hamburg, Germany) and anti-Flag-antibodies were from Sigma (Deisenhofen, Germany).

Reagents were obtained as follows: PMA, AEBSF, pepstatin A, Con A, ethylenediaminetetraacetic acid and ionomycin from Sigma, MCD from Research Biochemicals International (Natick, MA, USA), GM6001, TAPI-1 from Calbiochem. The hydroxamate based inhibitors GW280264X and GI254023X were described elsewhere.[23] The cloning of mADAM10 in pcDNA3.1 (Invitrogen, Karlsruhe, Germany) was reported previously.[24] ADAM17 was kindly provided by Carl Blobel (NY, USA). A HA-tag was introduced at the 3'-terminus of ADAM10 and ADAM17, respectively.

Cell culture and transfection. 293T cells, MEFs from ADAM9$^{-/-}$, ADAM10$^{-/-}$, ADAM15$^{-/-}$, ADAM17$^{-/-}$ mice and respective wild-type animals were generated and characterized as described elsewhere.[20,38] All cells were grown in Dulbecco's modified Eagle's medium with high glucose (PAA Laboratories, Pasching, Austria), supplemented with antibiotics and 10% fetal calf serum (FCS). Cells were transfected with FuGENE 6 (Roche Applied Science, Mannheim, Germany) according to manufacturer's instructions. For expression of hFasL in MEFs and 293T cells, the culture medium was supplemented with 40 μM zVad-fmk (Bachem, Weil am Rhein, Germany). The E6-1 Jurkat T cell line and CD4$^+$ T cell clones were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with glutamine, antibiotics, and 5% FCS. PBMC were isolated from buffy coat preparations from healthy volunteers and stimulated with PHA (0.5 μg/ml) for 4 days. Viable cells were further propagated in RPMI 1640 in the presence of rIL-2 (10 U/ml). To test the production of FasL in the presence or absence of inhibitors, T-cell blasts were washed once with phosphate-buffered saline (PBS) and incubated with the indicated substances for 1 h in RPMI 1640 before exposure to 5 μg/ml of Con A for polyclonal restimulation. After 12 h, culture supernatants were collected and tested for sFasL by ELISA. CD4$^+$ T-cell clones were prepared as described previously.[27] All clones and cell lines were established from PBMC of healthy donors. Initially, individual subpopulations were stimulated by PHA or SEA before cloning by limiting dilution. Superantigen-reactive $\alpha\beta$TCR clones were established by primary stimulation with SEA and cloning in the presence of SEA, allogeneic feeder cells (irradiated PBMC and LCL at a ratio of 10:1) and rhuIL-2. The SEA-reactive clones used are CD4-positive and do not react, e.g. to SEE (Supplementary Figure 3).

FasL can also be released through secretion of FasL-bearing microvesicles.[22] To exclude the detection of FasL released in exosomes into the culture medium, cell supernatants were subjected to ultracentrifugation before immunoblot or ELISA analyses.

FasL *in vitro* cleavage. Human recombinant FasL (250 ng/30 μl reaction mixture) containing the extracellular FasL domain (aa 103–281) fused at the N-terminus to a linker peptide (26 aa) and a Flag–tag (Alexis Biochemicals, Heidelberg, Germany) was incubated with recombinant human His-tagged ADAM10 (R&D Systems, Minneapolis, MN, USA) (250 ng/30 μl reaction mixture) for 14 h at 37°C. As control for unspecific degradation, FasL was also incubated alone in ADAM10 reaction buffer (25 mM Tris, 0.005% Brij-35; 2.5 μM $ZnCl_2$, pH 8.8) for the same period. Before starting the reaction, a sample was taken, which represents the intact FasL. Samples were mixed immediately with Laemmli buffer and were analyzed on Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). ADAM10 and FasL were analyzed by immunoblot with anti-His antibodies and anti-Flag antibodies (FasL).

Western blotting. Western blotting was performed as described elsewhere.[21] Briefly, cells were lysed in Triton lysis buffer. For analysis of sFasL the conditioned medium was subjected to ultracentrifugation (100 000 $\times$ *g*) for 2 h. Equal amounts of protein were loaded on 10% SDS gels or 12% Bis–Tris NuPAGE gels (Invitrogen) and were electrotransferred onto polyvinylidene fluoride-membranes (Hybond-P; Amersham, Braunschweig, Germany). Primary antibodies were detected using peroxidase–conjugated secondary antibodies.

FasL-specific ELISA. For analysis of sFasL the conditioned medium was subjected to ultracentrifugation (100 000 $\times$ *g*) for 2 h. The cell pellets were lysed in Triton lysis buffer (1% Triton-X-100, 5 mM Tris, 1 mM ethylene glycol tetraacetic acid, 250 mM saccharose, pH 7.4). As the buffer slightly reduced antibody/epitope binding ($<$10%), FasL standard for the analyses of cell pellets was also dissolved in Triton buffer. The FasL standard for the analysis of supernatants was dissolved in cell culture medium. The hFasL ELISA (R&D Systems, Minneapolis, MN, USA) was carried out according to manufacturer's instructions. Briefly, a 96-well plate (Microlon; Greiner, Frickenhausen, Germany) was coated over night with 2 μg/ml mouse anti-FasL capture antibody, subsequently washed three times with PBS with 0.05% Tween (PBS-T), and blocked with PBS-T containing 1% bovine serum albumin (BSA) for 1 h. Samples were added and the plate was incubated at room temperature (RT) for 2 h. Following washing, 50 ng/ml biotinylated anti-FasL

detection antibody in PBS-T containing 1% BSA was added to each well and the plate was incubated at RT for 1 h. After washing, 100 mU/ml streptavidin–peroxidase conjugate (Roche) in PBS-T containing 1% BSA was added followed by 20 min of incubation at RT. After washing, chromogenic POD substrate (BM Blue; Roche, Mannheim, Germany) was added. The reaction was stopped after 20 min of incubation at RT by addition of 2N H_2SO_4 before the optical density was determined at 450 nm, corrected for absorbtion at 540 nm.

FasL surface expression in cloned T lymphocytes. The kinetics of FasL expression were analyzed as described in detail before.[27] Briefly, 1×10^6 cloned T cells were incubated in medium or in the presence of MMP inhibitors at 37°C for 30 min. The cells were then stimulated with 10 ng/ml PMA and 500 ng/ml ionomycin. At the end of the indicated incubation time, the cells were transferred to a V-bottom 96-well plate, centrifuged, washed, and stained with the PE-conjugated anti-FasL-mAb. After 20 min, the cells were washed twice, fixed in paraformaldehyde and analyzed on a FACScan flow-cytometer using CellQuest™ analysis software (BD-Biosciences, Heidelberg, Germany). To correlate FasL surface expression and cleavage in the same experimental setting, 2.5×10^6 cloned $CD4^+$ T cells were pretreated in a 24-well plate with the different inhibitors for 1 h. The cells were then stimulated with PMA and ionomycin.

Small interfering RNA (siRNA). For downregulation of endogenous ADAM10 expression in human T cells (PHA blasts) the following RNAi oligonucleotides (Stealth RNAi, Invitrogen) were used: HSS100165, HSS100166 HSS100167. As a negative control, unspecific RNAi with low-GC content was used (Stealth™ RNAi Negative Control LO GC (12935–200)). For transfection experiments 5×10^6 cells were transfected with a total of 150 pmol of an equimolar mixture of the three constructs, using the AMAXA electroporation system (Program X-01) according to manufacturer's instructions. After 6 h of incubation the medium was replaced and the cells were further incubated for 72 h. After restimulation with PMA/IO, as described earlier, the conditioned media were harvested at different time points and released FasL was measured by ELISA. Subsequently, efficiency of ADAM10 knock down was measured by immunoblot.

Measurement of AICD. In the presence or absence of MMP inhibitors, SEA-reactive cloned human T cells were left unstimulated or stimulated with SEA (0.5 ng/ml) for 18 h. The ratio of dead/viable cells was analyzed by flow cytometry using PI (Sigma, 1 μg/ml in PBS) based on scatter and fluorescence properties. In parallel, FasL surface expression was tested with PE-conjugated NOK-1 using PE-conjugated mouse IgG1 as a control. Gates were set on the total or the viable cell population, respectively.

Chromium release assay. Target cells (Jurkat) were radiolabeled with 100 μCi $Na^2[^{51}Cr]O^4$ (Amersham-Buchler, Braunschweig, Germany), and washed three times. 1×10^4 cells were used for each assay. Each experimental condition was set up in triplicates and chromium release was determined by scintillation counting. Percentage of specific lysis was determined as 100 × (cpm experimental wells – cpm spontaneous release)/(cpm maximum release – cpm spontaneous release). The spontaneous release was routinely $<10\%$ of the maximum release.

Statistical analysis. Results are expressed as mean of triplicates $\pm$ S.D. or as means $\pm$ standard error of the mean (S.E.M.), as indicated respectively. The S.E.M. values indicate the variation between mean values obtained from at least three independent experiments. The assumptions for normality (Kolmogorov–Smirnov test) and equal variance (Levene median test) were verified with the SigmaStat 3.1 software (Erkrath, SYSSTAT, Germany). The analysis of variance were performed with one-way analysis of variance. Pairwise comparison procedures were performed with Tukey's test. A *P*-value less than 0.05 was considered to be significant.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)