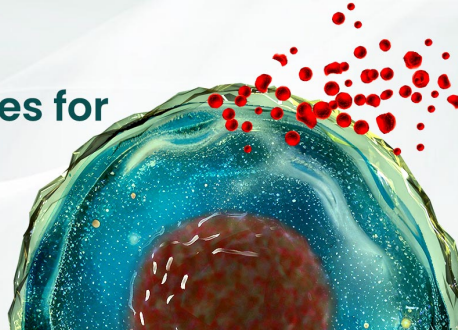




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## Cutting Edge: TNF- $\alpha$ -Converting Enzyme (TACE/ADAM17) Inactivation in Mouse Myeloid Cells Prevents Lethality from Endotoxin Shock<sup>1</sup>

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*TNF- $\alpha$ , a potent proinflammatory cytokine, is synthesized as a membrane-anchored precursor and proteolytically released from cells. Soluble TNF is the primary mediator of pathologies such as rheumatoid arthritis, Crohn's disease, and endotoxin shock. The TNF- $\alpha$ -converting enzyme (TACE), a disintegrin and metalloprotease 17 (ADAM17), has emerged as the best candidate TNF sheddase, but other proteinases can also release TNF. Because TACE-deficient mice die shortly after birth, we generated conditional TACE-deficient mice to address whether TACE is the relevant sheddase for TNF in adult mice. In this study, we report that TACE inactivation in myeloid cells or temporal inactivation at 6 wk offers strong protection from endotoxin shock lethality in mice by preventing increased TNF serum levels. These findings corroborate that TACE is the major endotoxin-stimulated TNF sheddase in mouse myeloid cells in vivo, thereby further validating TACE as a principal target for the treatment of TNF-dependent pathologies. The Journal of Immunology, 2007, 179: 2686–2689.*

The proinflammatory cytokine TNF- $\alpha$  has potent beneficial and detrimental functions (1). Its dysregulation triggers autoimmune diseases such as rheumatoid arthritis and Crohn's disease, but TNF is also critical for host defense against pathogens such as listeria. TNF is synthesized as a membrane-anchored precursor that is released by the TNF- $\alpha$ -converting enzyme (TACE)<sup>3</sup> a disintegrin and metalloprotease (ADAM) 17 (ADAM17) (2, 3). However, other enzymes, including ADAM10 (4), ADAM19 (5), matrix metalloproteinase 7 (6), and proteinase 3 (7) have also been implicated in releasing TNF, raising questions about the identity of the relevant TNF convertase in primary cells in vivo. LPS-triggered endotoxin

shock in mice, a model for septic shock, provides an excellent means to evaluate the mechanism and consequences of TNF processing in vivo (8–10). LPS stimulates TNF release via TLRs and the resulting endotoxin shock requires soluble, but not membrane-anchored, TNF (8) released from myeloid cells (9) and can be prevented with metalloprotease inhibitors (10). This has led to the hypothesis that TACE is the critical enzyme for endotoxin-triggered release of TNF from myeloid cells in vivo in mice. However, to date this hypothesis could not be tested because TACE-deficient mice die shortly after birth (11, 12).

To address the role of TACE in endotoxin shock in vivo, we generated conditional TACE-deficient mice. We found that the temporal systemic deletion of *Tace* by the inducible *Mx1-Cre* transgene (13) or its deletion in myeloid cells by the M lysozyme promoter (*LysM-Cre*) (14) protected the mice from endotoxin shock with serum TNF levels that were significantly lower than those in control animals. These observations corroborate the hypothesis that endotoxin shock in mice is caused by TACE-dependent shedding of TNF from myeloid cells.

### Materials and Methods

#### Generation of TACE conditional knockout mice

Genomic 129/SVJ DNA was used to generate a 1.6-kb PCR product with a noncoding sequence 1.1 kb upstream of exon 2 and a 5.2-kb PCR product with exon 2 and the surrounding noncoding region, which were subcloned into pCR2.1 TOPO vectors (Invitrogen Life Technologies). A loxP and an *EcoRI* site were inserted 1.0 kb downstream of exon 2, and the long and short arms were subcloned into a pKOII vector (provided by Dr. R. DePinho, Dana-Farber Cancer Center, Boston, MA). 129P2/OlaHsd cell clones harboring floxed *Tace* were identified by Southern blot and PCR analysis (Fig. 1, A and B). Chimeric mice were generated by C57BL/6 blastocyst injection and bred with C57BL/6 mice to generate floxed *Tace* heterozygous mice (referred to as *Tace*<sup>fllox/+</sup>; formal allele nomenclature: *Adam17*<sup>tm1Bbl</sup>). Homozygous *Tace*<sup>fllox/fllox</sup> mice were viable and fertile and did not display any evident pathological phenotype or histopathological defects (data not shown). *Tace*<sup>fllox/+</sup> mice were mated with *Tg(Ella-cre)C5379Lmgd* transgenic mice (15) (referred

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<sup>3</sup> Abbreviations used in this paper: TACE, TNF- $\alpha$ -converting enzyme; ADAM, a disintegrin and metalloprotease; mEF, mouse embryonic fibroblast; pIpC, polyinosinic-polycytidylic acid.

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to as *Ella-Cre*) to generate *Tace*-null heterozygous mice (referred to as *Tace*<sup>+/-</sup>; formal allele nomenclature: *Adam17*<sup>tm1.1Bbl</sup>) or with *Lyz2*<sup>tm1(Cre)Jfo</sup> (referred to as *LysM-Cre*) or *Tg(Mx1-cre)1Cgn* (referred to as *Mx1-Cre*) transgenic mice (13, 14) to generate conditional TACE-deficient mice. For temporal deletion of floxed *Tace*, 6-wk-old *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> mice were injected i.p. with 250 μg of polyinosinic-polycytidylic acid (pIpC; Sigma-Aldrich) three times at 2-day intervals as described (13). In all experiments with *Tace*<sup>flox/flox</sup>/*Cre*<sup>+</sup> mice, littermate *Tace*<sup>flox/flox</sup>/*Cre*<sup>-</sup> mice served as controls.

### Histology

Tissues were fixed in 4% paraformaldehyde and PBS, sectioned, stained with H&E, and photographed with a DXM1200 camera (Nikon) on a BX50 microscope (Olympus). Images were processed with Adobe Photoshop CS2.

### Generation of immortalized mouse embryonic fibroblasts

Embryonic fibroblasts isolated from E13.5 *Tace*<sup>-/-</sup> embryos as described (16) were immortalized with the SV40 large T Ag. Absence of TACE protein in *Tace*<sup>-/-</sup> fibroblasts was confirmed by Western blotting (see Fig. 1C).

### Shedding assay

Immortalized fibroblasts were transfected with alkaline phosphatase-tagged TNF or TGFα (see Ref. 16 for details). Fresh Opti-MEM (Invitrogen Life Technologies) with or without 25 ng/ml PMA was added for 1 h after 1 day, and alkaline phosphatase activity was measured by colorimetry or visualized by in-gel staining of substrates as described (16).

### Production of TNF by macrophages in vitro

Bone marrow was collected from the tibiae and femurs of 6-wk-old *Tace*<sup>flox/flox</sup>/*LysM-Cre*<sup>-</sup> or littermate *Tace*<sup>flox/flox</sup>/*LysM-Cre*<sup>+</sup> mice. RBCs were removed with RBC lysis buffer (Roche), and the remaining cells were plated on tissue culture plates. Adherent cells were grown in DMEM with 10% FCS, antibiotics, and 50 ng/ml recombinant mouse macrophage CSF (WAKO) for 4 days and then used as bone marrow macrophages and incubated with or without 1 μg/ml LPS (Sigma-Aldrich) for 3 h. Soluble TNF was measured by ELISA (R&D Systems).

### Endotoxin shock

Endotoxin shock was induced by i.p. injection of 5 μg of LPS (Sigma-Aldrich) and 20 mg of D-galactosamine (WAKO). All injected mice were closely monitored every hour for the first 16 h and every 3–6 h thereafter. To enhance serum TNF levels in a separate experiment (see Fig. 3C), *Tace*<sup>flox/flox</sup>/*LysM-Cre*<sup>-</sup> or littermate *Tace*<sup>flox/flox</sup>/*LysM-Cre*<sup>+</sup> mice were treated with 100 μg of LPS and 20 mg of D-galactosamine and sacrificed after 3 h. Serum TNF was measured by ELISA. All animal experiments were approved by the Institutional Animal Care and Use Committee of the Keio University School of Medicine (Tokyo, Japan).

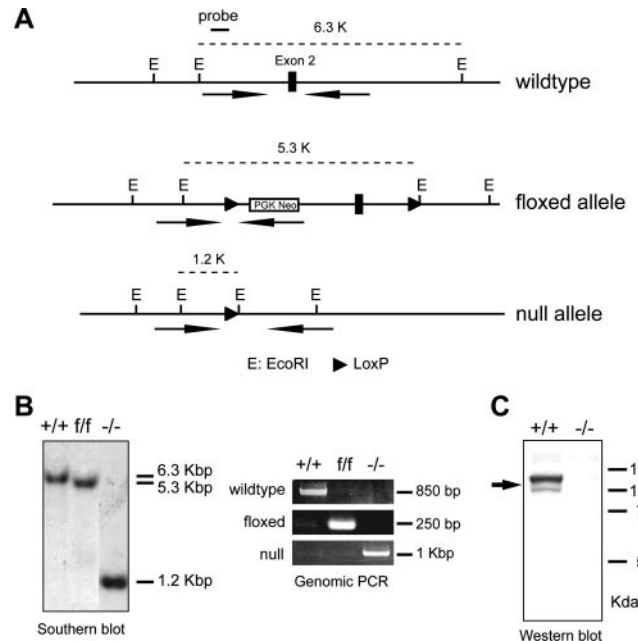
### Statistical analysis

A Student's *t* test for two samples assuming equal variances was used to calculate the *p* values. *p* < 0.05 was considered statistically significant.

## Results and Discussion

### Generation and analysis of *Tace*<sup>flox/flox</sup> and *Tace*<sup>-/-</sup> mice

Mice containing a floxed *Tace* allele were crossed with a germline “Cre-deleter” strain (*Ella-Cre*) (15) to generate *Tace*<sup>+/-</sup> animals that were then bred to produce *Tace*<sup>-/-</sup> mice (Fig. 1A; see *Materials and Methods* for details). Removal of floxed *Tace* was confirmed by PCR and Southern blotting (Fig. 1B) and the lack of TACE protein was corroborated by Western blotting (Fig. 1C). The *Tace*<sup>-/-</sup> animals generated in this study were indistinguishable from the previously described *Tace*<sup>ΔZn/ΔZn</sup> mice that lack the exon carrying the catalytic site of TACE (2, 11); they died shortly after birth (Table I), with open eyes and defects in the aortic, pulmonic, and tricuspid heart valves (Fig. 2, A and B; pulmonic valves not shown). In addition, constitutive and phorbol ester-stimulated shedding of transfected TGF-α and TNF was strongly reduced in *Tace*<sup>-/-</sup> mouse embryonic fibroblasts (mEF) compared with wild-type controls (Fig. 2C) as previously described for *Tace*<sup>ΔZn/ΔZn</sup> mEF (5, 11, 16). Thus, Cre-mediated excision of floxed *Tace* results in the inactivation of TACE.



**FIGURE 1.** Generation of *Tace*<sup>flox/flox</sup> and *Tace*<sup>-/-</sup> mice. *A*, Schema for conditional targeting of *Tace*. E indicates *EcoRI* site and arrowheads indicate loxP sites placed surrounding exon 2 and PGK neo. Arrows indicate primers used for genotyping. *B*, Southern blotting and PCR analysis of genomic DNA isolated from wild-type (+/+), floxed *Tace* (*f/f*), and *Tace* null (-/-) mice. *C*, TACE Western blot of lysates of mouse embryonic fibroblasts from embryonic day 13.5 wild-type and *Tace*<sup>-/-</sup> embryos.

### Temporal TACE disruption by *Mx1-Cre* prevents lethality from endotoxin shock

To evaluate the role of TACE in adult mice, we generated *Tace*<sup>flox/flox</sup>/*Mx1-Cre* animals to allow excision of the floxed exon by i.p. injection of pIpC (13) in 6-wk-old mice. The pIpC-induced recombination in *Mx1-Cre* mice occurs in various organs with different efficiency, with almost complete recombination in bone marrow, liver, and spleen (13). Accordingly, we found strongly decreased amounts of floxed TACE in these tissues in *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> mice 1 wk after the pIpC injection (Fig. 3A). After treatment with pIpC, *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> and *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>-</sup> mice displayed no evident pathological phenotypes or histopathological defects for at least 2 wk (data not shown). We next tested the survival of *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> animals in a murine model for endotoxin shock, which depends on soluble TNF in vivo (8). When *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> mice or *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>-</sup> controls were injected i.p. with LPS and D-galactosamine, six of eight *Mx1-Cre*<sup>+</sup> mice were protected from endotoxin shock whereas seven of eight *Mx1-Cre*<sup>-</sup> controls died within the first 16 h after injection (Fig. 3B). Serum TNF levels after LPS injection were also significantly lower in *Tace*<sup>flox/flox</sup>/*Mx1-Cre*<sup>+</sup> mice (230 pg/ml, ± 177 SD; *n* = 7) compared with wild-type controls (852 pg/ml, ± 349 SD; *n* = 8) or *Tace*<sup>flox/flox</sup>/*Cre*<sup>-</sup> animals (779 pg/ml, ± 310 SD; *n* = 10) (Fig. 3C). These results support the hypothesis that the shedding of TNF by TACE is crucial for LPS-induced endotoxin shock in mice, although protection from endotoxin shock could also be due, at least in part, to the reduction in cleavage of other TACE substrates.

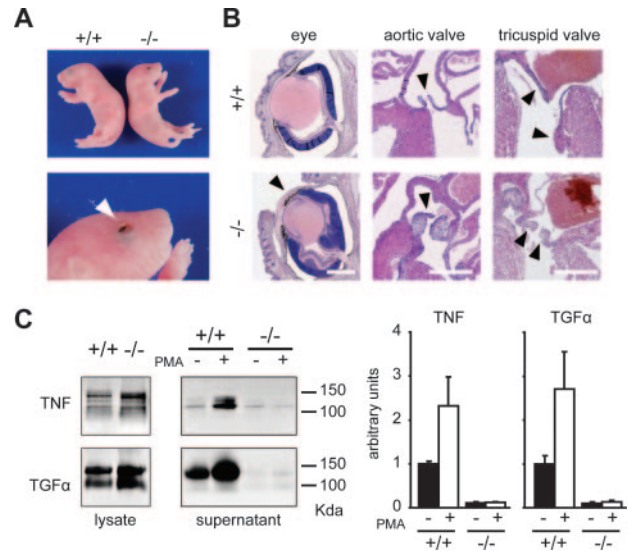
Table I. Mendelian distribution of offspring from  $Tace^{+/-} \times Tace^{+/-}$  mating

Age <sup>a</sup>	Total	+/+	+/-	-/-
E17.5–18.5	87	23	45	19
P1–2	87	28	49	10
P13–15	78	26	52	0

<sup>a</sup>E, Embryonic; P, postnatal.

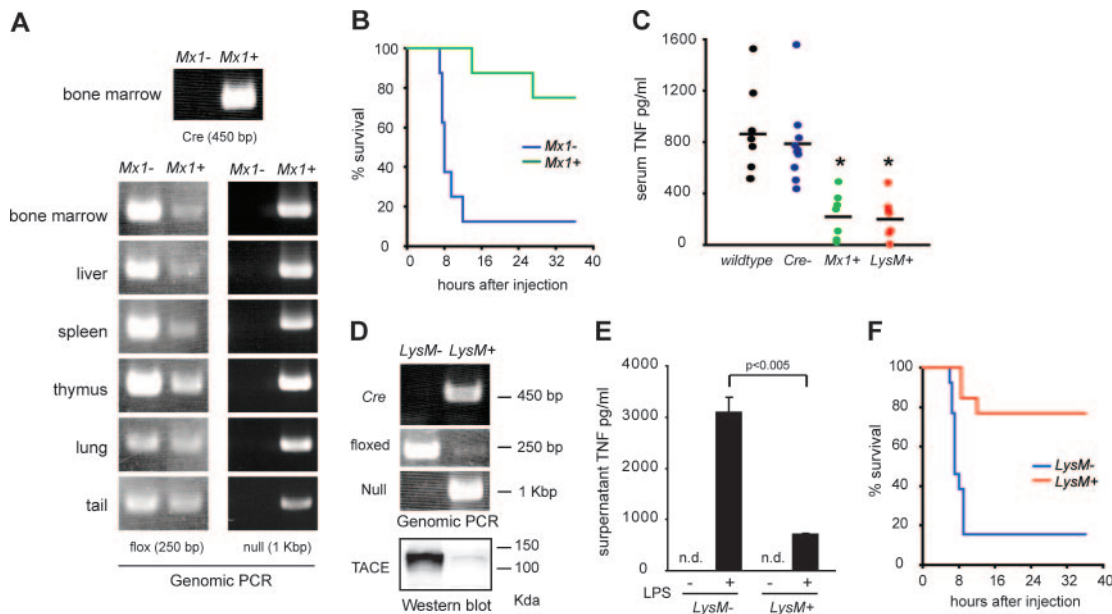
### Inactivation of TACE in myeloid cells protects from endotoxin shock

To further narrow down the cell type in which TACE is required for endotoxin-induced release of TNF and the resulting lethality, we generated  $Tace^{flox/flox}/LysM-Cre^{+}$  mice to remove TACE in myeloid cells (14), which are critical for LPS-induced shock (9).  $Tace^{flox/flox}/LysM-Cre^{+}$  mice were viable and fertile and did not display any evident abnormalities (data not shown). Macrophages isolated from  $Tace^{flox/flox}/LysM-Cre^{+}$  mice had very low amounts of the floxed *Tace* allele, as confirmed by PCR, and of the TACE protein, as detected by Western blot analysis, compared with the  $Tace^{flox/flox}/LysM-Cre^{-}$  controls (Fig. 3D). LPS-stimulated release of soluble TNF was strongly reduced in macrophages isolated from  $Tace^{flox/flox}/LysM-Cre^{+}$  animals compared with  $Tace^{flox/flox}/LysM-Cre^{-}$  controls (Fig. 3E), which is consistent with the recently reported ablation of TNF shedding from  $Tace^{\Delta Zn/\Delta Zn}$  leukocytes isolated from a radiation chimera (17). When  $Tace^{flox/flox}/LysM-Cre^{+}$  animals and  $Tace^{flox/flox}/LysM-Cre^{-}$  controls were subjected to LPS and D-galactosamine to trigger endotoxin shock, 11 of 13 control animals perished within 8 h of injection whereas only three of 13  $Tace^{flox/flox}/LysM-Cre^{+}$  animals succumbed to this challenge



**FIGURE 2.** Characterization of  $Tace^{-/-}$  mice. *A*, Newborn  $Tace^{-/-}$  ( $-/-$ ) and wild-type ( $+/+$ ) mice (upper panel);  $Tace^{-/-}$  embryos have open eyes (arrowhead, lower panel). *B*, Histological analysis of the eyes and the aortic and tricuspid heart valves of newborn wild-type and  $Tace^{-/-}$  mice (arrowheads). Bar, 500  $\mu$ m. *C*, Evaluation of TNF and TGF- $\alpha$  shedding from immortalized wild-type and  $Tace^{-/-}$  mEFs in the presence or absence of the phorbol ester PMA. An in-gel assay of substrate expression in lysates and shedding into the supernatant is shown in the left panel and a colorimetric assay of shedding into the supernatant is shown in the right panel (see Materials and Methods for details).

(Fig. 3F). Following endotoxin challenge, the average TNF serum levels in  $Tace^{flox/flox}/LysM-Cre^{+}$  (212 pg/ml,  $\pm$  158 SD;  $n = 7$ ) were very similar to the levels in  $Tace^{flox/flox}/Mx1-Cre^{+}$



**FIGURE 3.** Temporal ablation of *Tace* or ablation in myeloid cells prevents lethality from LPS-induced shock. *A*, The efficiency of Cre-induced *Tace* excision in different organs in  $Tace^{flox/flox}/Mx1-Cre^{+}$  ( $Mx1^{+}$ ) mice was evaluated by PCR using comparable amounts of genomic DNA from  $Mx1-Cre^{+}$  and  $Mx1-Cre^{-}$  ( $Mx^{-}$ )  $Tace^{flox/flox}$  mice as template. *B*, Seven- to 9-wk-old  $Tace^{flox/flox}/Mx1-Cre^{-}$  or  $Tace^{flox/flox}/Mx1-Cre^{+}$  mice treated with pIpC were injected i.p. with 5  $\mu$ g LPS and 20 mg D-galactosamine and monitored for survival up to 36 h ( $n = 8$ ). *C*, Systemic TNF production induced by LPS. LPS (100  $\mu$ g) was injected i.p. into wild-type,  $Tace^{flox/flox}/Cre^{-}$  ( $Cre^{-}$ ),  $Tace^{flox/flox}/Mx1-Cre^{+}$  pIpC-treated ( $Mx1^{+}$ ) or  $Tace^{flox/flox}/LysM-Cre^{+}$  ( $LysM^{+}$ ) mice, sera were collected 3 h after injection, and TNF was measured by ELISA. \*,  $p < 0.005$  between  $Mx1^{+}$  or  $LysM^{+}$  and wild-type or  $Cre^{-}$ . *D*, PCR analysis and Western blotting of cell lysates from  $Tace^{flox/flox}/LysM-Cre^{-}$  and  $Tace^{flox/flox}/LysM-Cre^{+}$  bone marrow macrophages. *E*, LPS-stimulated TNF secretion from  $Tace^{flox/flox}/LysM-Cre^{-}$  and  $Tace^{flox/flox}/LysM-Cre^{+}$  bone marrow-derived macrophages. LPS (1  $\mu$ g/ml) was added to bone marrow macrophages isolated from 6-wk-old wild-type  $Tace^{flox/flox}/LysM-Cre^{-}$  or  $Tace^{flox/flox}/LysM-Cre^{+}$  mice and incubated for 3 h and TNF was measured by ELISA. n.d., Not detectable. *F*, Seven- to 9-wk-old mice were injected i.p. with 5  $\mu$ g of LPS and 20 mg of D-galactosamine and monitored for survival up to 36 h ( $n = 13$ ).

mice (230 pg/ml,  $\pm$  177 SD;  $n = 7$ ) and, thus, significantly lower than in *Tace<sup>fllox/fllox</sup>/Cre<sup>-</sup>* animals or wild-type controls (see above and Fig. 3C). The remaining endotoxin-stimulated shedding of TNF in *Tace<sup>fllox/fllox</sup>/LysM-Cre<sup>+</sup>* and *Tace<sup>fllox/fllox</sup>/Mx-Cre<sup>+</sup>* is most likely due to cells in which one or both floxed *Tace* alleles escaped Cre excision (see Fig. 3, A and D), although it cannot be ruled out that other candidate TNF sheddases also contribute to TNF shedding following Cre excision of TACE.

Taken together, these results provide strong evidence that TACE is indeed a principal enzyme responsible for the release of soluble TNF from the relevant primary cells (myeloid cells) during endotoxin shock in an intact organism in vivo (a discussion of criteria to link enzymes and relevant substrates can be found in Ref. 18). These results are consistent with studies of mice lacking an endogenous inhibitor of TACE, the tissue inhibitor of matrix metalloproteinases 3 (TIMP3) (19), that have an exacerbated response to an endotoxin challenge (20). In addition to releasing TNF, TACE is known to have a role in the shedding of numerous other membrane proteins from mEFs (11, 16, 21), and TACE activation in leukocytes also triggers shedding of the TNF receptors I and II (17). Shedding of these and other membrane anchored cytokines and growth factors could thus potentially contribute to the anergic state of the immune system at later stages of septic shock. It would therefore be interesting to determine whether or not TACE inhibitors might offer advantages in the treatment of septic shock in humans over the blocking of TNF, at least in those cases where an involvement of TNF is likely (reviewed in (22)). Moreover, our results further support the notion that TACE is likely also a good target for treatment of other pathologies caused by soluble TNF, most notably rheumatoid arthritis, Crohn's disease, and psoriasis. The *Tace<sup>fllox/fllox</sup>* animals described here will help address these questions, and permit an evaluation of other TACE functions during mouse development and in disease models, including those related to the role of TACE in epidermal growth factor receptor-ligand activation in cancer (23).

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## Disclosures

The authors have no financial conflict of interest.

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