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The immunoregulator soluble TACI is released by ADAM10 and reflects B-cell activation in autoimmunity

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Abstract

BAFF and APRIL, which control B-cell homeostasis, are therapeutic targets in autoimmune diseases. TACI-Fc (atacicept), a soluble fusion protein containing the extracellular domain of the

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BAFF-APRIL-receptor TACI, was applied in clinical trials. However disease activity in multiple sclerosis (MS) unexpectedly increased, whereas in systemic lupus erythematosus (SLE) atacicept was beneficial. Here, we show that an endogenous soluble form of TACI exists in vivo. TACI proteolysis involved shedding by ADAM10 releasing sTACI from activated B cells. The membrane-bound stub was subsequently cleaved by γ-secretase reducing ligand-independent signaling of the remaining C-terminal fragment. The shed ectodomain assembled ligand-independently in a homotypic way. It functioned as a decoy receptor inhibiting BAFF- and APRIL-mediated B-cell survival and NFκB-activation. We determined sTACI levels in autoimmune diseases with established hyper-activation of the BAFF-APRIL system. sTACI levels were elevated both in the cerebrospinal fluid (CSF) of the brain-restricted autoimmune disease MS correlating with intrathecal IgG production, as well as in the serum of the systemic autoimmune disease SLE correlating with disease activity. Together, we show that TACI is sequentially processed by ADAM10 and γ-secretase. The released sTACI is an immunoregulator that shares decoy functions with atacicept. Itreflectssystemic and compartmentalized B-cell accumulation and activation.

Introduction

B cells play a significant role in the pathogenesis of autoimmunity and B-cell modulating therapies are promising in the treatment of a variety of autoimmune diseases(1). Regulation of B-cell homeostasis involves the BAFF-APRIL system that is comprised of two ligands, *B-cell activating factor of the TNF-family* (BAFF) and *a proliferation inducing ligand*(APRIL), and three receptors, *B-cell maturation antigen* (BCMA), *transmembrane activator and CAML interact or* (TACI), and *BAFF-receptor* (BAFF-R) (2).

In systemic lupus erythematosus (SLE) an involvement of the BAFF-APRIL axis is prominent, as mice over expressing BAFF develop an SLE-like phenotype, BAFF is elevated in the serum of SLE patients, and the monoclonal antibody (mAb) belimumab targeting BAFF is beneficial in a proportion of SLE patients (3, 4). In multiple sclerosis (MS), an organ-specific autoimmune disease characterized by local Ig production with long-term persistence of B cells in the CNS (5, 6), BAFF is up regulated in MS plaques and is produced by astrocytes (7). While depletion of B cells in MS with anti-CD20 antibodies is promising (8), targeting the B-cell survival factors BAFF and APRIL with atacicept, a recombinant fusion protein containing the extracellular ligand-binding portion of TACI linked to the Fc domain of human IgG, unexpectedly increased disease activity in MS patients (9), whereas in SLE atacicept was beneficial at least at a high dose(10).

TACI is a type-I oriented transmembrane protein belonging to the TNF-receptor (TNFR) super family. It is expressed on $CD27^+$ memory B cells, plasma cells and a subpopulation of $CD27^-$ B cells and is induced early upon B-cell activation (11). The ectodomain of TACI contains two cysteine-rich domains (CRD). The first CRD is involved in ligand-independent assembly of TACI into multimeric complexes, while the second CRD is required for binding of BAFF and APRIL(12). Ligand binding to TACI recruits signaling molecules to the intracellular domain of TACI, which leads to activation of nuclear factor of activated T-cells (NFAT) and NFκB (13, 14). Studies of TACI−/− mice showed that this receptor is both a

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positive and a negative regulator of B-cell responses (15–17). Mutations in TACI are a cause of common variable immunodeficiency (CVID) and IgA deficiency (18, 19).However, some of these patients in addition develop signs of autoimmunity and lymphoproliferation(19).

Importantly, the functions of some transmembrane receptors extend beyond signal transmission, as they can be processed into soluble receptors (20).Here, proteases of the *A disintegrin and metalloproteinase*(ADAM) family are involved in ectodomain shedding of a variety of membrane proteins (21). This can modulate signaling activity either by down-regulation of membrane-bound receptors or by the release of soluble receptors like sTNFR1(22) or sIL6-R (23). In the case of type I transmembrane proteins, the γ-secretase complex may further cleave the remaining fragment within the plasma membrane(24) in a process called regulated intramembrane proteolysis (RIP)(25).

Here, we show that the TACI extracellular domain is shed from activated B cells by ADAM10, giving rise to soluble TACI (sTACI). The remaining C-terminal fragment (CTF) is cleaved by γ-secretase. sTACI assembles homotypically; it binds BAFF and APRIL to block NFκB-activation and B-cell survival. In systemic (SLE) and compartmentalized (MS) immunopathologies we detected elevated levels of sTACI establishing sTACI as a potential biomarker.

Materials and Methods

Patients

All patient samples were collected following written informed consent according to local ethics policy guidelines in Stockholm, Berlin and Munich and the Declaration of Helsinki. We analysed the following samples: CSF from 37 untreated MS patients (CIS: n=10, RR-MS: n=20, SP-MS: n=7) and from 20 untreated patients with ONDs (sensory symptoms: n=7, cerebrovascular disease: n=1, migraine: n=1, vertigo: n=1, syringomyelia: n=1, spinal stenosis: n=1, neurasthenia: n=1, alcohol-related spastic paraparesis: n=1, hearing deficit: n=1, depression and idiopathic pain: n=1, fatigue: n=1, bipolar disorder: n=1, schizophrenia: n=1, diplopia: n=1); plasma from 57 untreated MS patients (CIS: n=18, RR-MS: n=23, SP-MS: n=16) and 18 untreated patients with other neurological diseases (ONDs); CSF from 25 MS patients before and 12 months after treatment with natalizumab; serum from 17 untreated SLE patients and 22 treated SLE patients (treatment included prednisolone, chloroquine, azathioprine, cyclophosphamide, mycophenolate mofetil, methotrexate); serum samples of 10 patients prior and after treatment with corticosteroids; serum from 33 healthy volunteers. Intrathecal IgG production was calculated as IgG-index ((CSF IgG/ CSF albumin)/ (serum IgG/ serum albumin)).

Plasmids

Human TACI in pCMV6-XL4, human BCMA in pCMV6-XL5 and the control vector pCMV6-AN-DDK were purchased from Origene Technologies. A C-terminal FLAG-tag, an N-terminal FLAG-tag and an N-terminal HA-tag were added to TACI in pcDNA3.1 using standard cloning procedures. To generate TACI-Δ-Ecto, the TACI sequence coding for amino acids 155–293 was cloned into pcDNA3.1. CRE followed by the T2A peptide

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sequence and GFP or GFP alone were cloned into the retroviral vector pMSCV. The pCL-Eco packaging construct was additionally used for retrovirus production.

Protease inhibitors

DAPT (Merck Calbiochem), TAPI-1 (Merck Calbiochem), C3 (Merck Calbiochem) and GI254023X (kindly provided by Dr. Andreas Ludwig, University of Erlangen, Germany) were used.

Cell culture

Raji cells were cultured in RPMI-1640 (Sigma-Aldrich)/ 10% FBS (Biochrom AG)/ 100 U/ml penicillin + 100 μg/ml streptomycin (Gibco, Invitrogen)/ 1% non-essential amino acids (Gibco, Invitrogen)/ 1 mM sodium-pyruvate (Gibco, Invitrogen)/ 2 mM L-glutamine (Pan Biotech). HEK293T cells were cultured in DMEM (Sigma-Aldrich)/ 10% FBS/ 100 U/ml penicillin + 100 μg/ml streptomycin.

Transient transfection

HEK293T cells were transfected using Lipofectamine 2000 (Invitrogen). sTACI containing and control supernatants were generated by transfection of 10 μg of TACI in pCMV-XL4 or an empty vector. After 3 d supernatants were harvested and centrifuged 2 × 400g for 7 min and 1 × 20.000g for 30 min.

Human B cell culture and stimulation

PBMCs were isolated by density gradient separation using Pancoll (Pan Biotech). Human B cells were isolated by negative selection using the EasySep™ Human B Cell Enrichment Kit (Stem Cell Technologies). Human B cells were seeded at 8×10^5 cells/ml and activated using ODN 2006 (2.5 μg/ml, Invivogen), anti-IgM (10 μg/ml Jackson ImmunoResearch), R848 (1 μg/ml, Sigma-Aldrich), recombinant human IL-2 (25 ng/ml, R&D Systems), co culture with CD40L expressing mouse L cells (5×10^4 cells/ml) and recombinant human IL-21 (50 ng/ml, EBioscience). Supernatants were collected after 4 d. sTACI production and IgG production were measured. TACI surface expression was determined using a PE mouse anti-human TACI antibody (5 μg/ml, Clone: FAB1741P, R&D Systems) and the corresponding PE mouse IgG1 isotype control (5 μg/ml, Clone: X40, Becton Dickinson).

Murine B cell culture and survival assay

For murine B-cell cultivation spleens from C57BL/6 mice (8–12 wk of age) were passed over a 40-micron cell strainer. Red cell lysis was performed using ACK buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM EDTA) for 2 minutes on ice. B cells were isolated using the EasySep™ Mouse B Cell Isolation Kit (Stem Cell Technologies) by negative selection. The murine B cells were seeded at a concentration of 3×10^6 cells/ml on cell culture plates, coated with anti-murine IgM antibodies (2.5 μg/ml) overnight at RT. Cells were treated with the indicated concentrations of HEK293T cell supernatants containing sTACI and control supernatants generated as described above. Additionally TACI-Fc was added (R&D Systems) and cells were stimulated with BAFF (100 ng/ml, R&D Systems) and APRIL (100 ng/ml, AdipoGen). After 2 d of cultivation mouse B cells were analysed by flow cytometry

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using a PE anti-mouse CD19 antibody (1 μg/ml, Clone: MB19-1, eBioscience) and APC TO-PRO®-3 Iodide (250nM, Life Technologies). PE positive and APC negative cells were determined.

Western blotting and immunoprecipitation

To analyse the role of γ-secretase, TACI with a C-terminal FLAG-tag was transfected into HEK293T cells using Lipofectamine 2000. Increasing amounts of DAPT were added. After 48 h cells were lysed in NP40 buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1% Nonidet P-40 and complete protease inhibitor cocktail (Roche Applied Science)). Proteins were electrophoresed on 10–20% Tricine gels, transferred to PVDF membranes, and blotted with an anti-FLAG antibody (Clone: M2, Sigma-Aldrich). To control for equal protein loading membranes were stained with anti-β-Actin antibody (Clone: C4, Santa Cruz Biotechnology). For immunoprecipitation of sTACI from HEK293T a monoclonal mouse anti-TACI antibody (Clone: MAB174, R&D Systems) or a mouse IgG1 control (R&D Systems) were used. For immunoprecipitation from Raji cell supernatant the polyclonal goat anti-TACI antibody (Clone: AF174; R&D Systems) or a goat IgG control (R&D Systems) were applied. Antibodies were coupled to Dynabeads® Protein G (Life Technologies) and cross-linked with bis-sulfosuccinimidyl-suberate (BS3) (Pierce Chemical Co.). sTACI derived from HEK293T cells was eluted with NuPAGE® LDS Sample Buffer (Life Technologies), electrophoresed on 4–12% Bis-Tris gels (Life Technologies) with MES-running buffer, blotted on PVDF membranes and stained with a monoclonal mouse anti-TACI antibody (Clone: MAB174, R&D Systems). Alternatively the PVDF membrane was cut prior to staining at the height corresponding to sTACI and N-terminal sequencing was performed. sTACI derived from RAJI cells was obtained by acidic elution. For co-immunoprecipitation of TACI-N-HA and TACI-N-FLAG, HEK293T cells were transfected with the respective expression constructs. 48h later cells and supernatants were harvested. Cells were lysed in IP buffer (0.5% NP-40, 50 mM HEPES, 250 mM NaCl, 5 mM EDTA and complete protease inhibitor cocktail (Roche Applied Science)). Immunoprecipitation was performed using anti-FLAG magnetic beads (Sigma-Aldrich). IP eluates and pre IP samples were subjected to ELISA.

Mass Spectrometry and Edman-sequencing

sTACI derived from Raji cells was digested in solution with trypsin or GluC; sTACI derived from HEK293T cells was digested with trypsin and chymotrypsin. Mass spectrometry (LTQ Orbitrap XL) was performed.

ELISA

Human sTACI and IgG concentrations were determined using the Human TACI Duo Set (DY174, R&D Systems) and the Human IgG ELISA development kit (Mabtech AB) respectively. Complex formation between sTACI and BAFF and APRIL was determined by coating anti-FLAG antibody (M2, 5 μg/ml, Sigma-Aldrich) on ELISA plates, incubating them with BAFF-FLAG (Enzo Life Sciences) and APRIL-FLAG (AdipoGen) (200 ng/ml) and adding sTACI and TACI-Fc (R&D Systems) (25 ng/ml). Bound TACI was detected using the TACI Duo Set ELISA Kit. To detect TACI-N-HA co-immunoprecipitated with

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TACI-N-FLAG, ELISA plates were coated with an anti-HA.11 (Covance) antibody (5 μg/ml). The following ELISA steps were performed using the TACI Duo Set ELISA Kit.

NFκB-reporter assay

HEK293T cells were cotransfected with a firefly luciferase reporter plasmid, the internal control CMV *Renilla* luciferase plasmid and the respective expression plasmids. The decoy-function of sTACI was assessed by adding BAFF or APRIL-FLAG (100 ng/ml) to supernatants containing sTACI or to control supernatants. After incubation at 37°C for 30 minutes, supernatants were added to the BCMA-transfected cells. 16 h later cells were lysed with passive lysis buffer (Promega) and reporter gene activity was determined using firefly luciferase substrate (Biozym) and *Renilla* luciferase substrate (Promega) respectively. To analyse the role of γ-secretase on NFκB-activation, TACI-Δ-Ekto or full-length TACI were transfected together with the above described luciferase plasmids. 8 h after transfection DAPT and BAFF were added. 16 h later NFκB-activation was measured as described.

Retrovirus production and transduction of B cells from ADAM10 cKO mice

We isolated splenic B cells from ADAM10 conditional knock-out mice (ADAM10 cKO, (26)) which contain two flox sites flanking the ADAM10 gene. Mouse B cells were stimulated for 2 d with CpG ODN 1668 20 μg/ml (Invivogen) and recombinant CD40L 2.5 μg/ml (R&D Systems). Retrovirus was produced as described previously (27) using the vector pMSCV expressing GFP or the CRE-recombinase followed by the sequence of the self-cleaving T2A peptide and GFP. B cells were spin-infected as described previously (27). On day 4 TACI expression was determined using a rat monoclonal anti-mouse TACI antibody (Clone: FAB1041A, 5 μg/ml, R&D Systems). Knock-down of ADAM10 was determined by Western Blot using a rabbit monoclonal anti-ADAM10 antibody (Clone: EPR5622, Abcam) in FACS-sorted GFP positive cells.

Lentiviral shRNA mediated knock-down

Lentivirus production was performed as described previously (28). shRNA sequences are listed in table S1. Raji cells were plated at 1×10^6 cells/ml and transfected with conditioned medium containing the lentiviruses. The transduced Raji cells were plated at 5×10^5 cells/ml. 24 h later supernatants were harvested and analysed for sTACI concentration by ELISA. TACI surface expression was determined by FACS using a mouse monoclonal anti-TACI antibody (Clone: MAB174, 1 μg/ml, R&D Systems) and the corresponding mouse IgG1 isotype control (R&D Systems).

Quantitative PCR

RNA from transduced Raji cells was isolated using the RNeasy Micro Kit (Qiagen). cDNA was generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). For real-time PCR TaqMan® assays for ADAM10, ADAM17 (Applied Biosystems) and PrimeTime® qPCR Assays for ADAM9 and ADAM19 (Integrated DNA Technologies) were used in combination with the TaqMan® PCR Core Reagent Kit (Applied Biosystems). Cyclophilin (Applied Biosystems) expression was determined as a housekeeping gene. Samples were run in MicroAmp™ Optical 96-well reaction plates

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(Applied Biosystems) in a 7900HT Fast Real-Time PCR System (Applied Biosystems). Data was analysed using SDSv2.3 software (Applied Biosystems).

Statistics

Statistical significance was assessed with Prism Software (GraphPad) by unpaired or paired, non-parametric or parametric t-test analysis or by spearman´s correlation, as appropriate. P-values of *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 were considered significant and designated accordingly.

Results

sTACI is released by activated B cells

As TACI is described as an ambivalent regulator of B-cell homeostasis, we speculated that this effect could be due to a soluble variant of TACI. Therefore we stimulated primary human B cells with a panel of different B-cell activators and determined plasma membrane expression of TACI (Fig. 1A) and in parallel release of sTACI (Fig 1B). While B cells in human blood expressed little TACI on their surface, we noted a strong induction by TLR7/8, TLR9 agonists and by CD40L, which was further enhanced by addition of IL-21 (Fig. 1A) (11). sTACI release and TACI surface expression, induced by the different stimuli,correlatedstrongly (Fig. 1C),suggesting that sTACI production by primary B cells parallels levels of TACI on the cell surface. In addition, we analysed whether sTACI release is linked to differentiation into Ig-secreting cells. We found that activated B cells released sTACI without producing IgG, e.g. after CD40L and R848 stimulation (Fig. 1B). To identify stimuli that trigger sTACI release from membrane-bound TACI, we stimulated the Burkitt lymphoma cell line Raji, which endogenously expresses TACI, with different TLR-ligands and cytokines (poly(I:C), CpG ODN 2006, TNF-α, IFN-γ, PMA). We did not observe astrong modulation of sTACI release(Fig. S1A), only PMA weakly and transiently induced TACI shedding (Fig. S1B). Thus, when TACI is present on the cell surface, it is shed without the need of an additional stimulus. Together, this suggests that sTACI release reflects TACI expression on the cell surface.

sTACI represents the extracellular part of TACI

Next we determined the molecular weight and amino acid composition of sTACI. We immunoprecipitated sTACI from the supernatant of HEK293T cells that had been transfected with full-length human TACI. sTACI had a molecular weight of ~ 13kDa in Western Blot analysis and silver staining (Fig. 2A). For mass spectrometry, we analysedthe band detected by silver staining and sTACI immunoprecipitated from Raji cell supernatant. After trypsin, chymotrypsin and GluCdigestion as well as N-terminal sequencing, we found soluble TACI to be composed of the extracellular part of TACI (Fig. 2B). The sequence at the C-terminus ended with K154, adjacent to L155 in sTACI derived from HEK293T cells and Raji cells (Fig. 2B). K154/L155 is within the juxtamembrane region and comprises a typical cleavage motif of metalloproteinases of the ADAM and membrane-type matrix metalloproteinases (MT-MMP) family(28). Therefore, K154/L155 could be the naturally occurring cleavage site of TACI resulting in generation of sTACI. Alternatively this site can also be recognized by trypsin.

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ADAM10 sheds TACI

To identify the protease that sheds TACI, we applied different protease inhibitors to Raji cells. We found that the metalloproteinase inhibitor TAPI-1, as well as GI254023X (29), a specific ADAM10 inhibitor, blocked sTACI release and increased surface expression of TACI (Fig. 3A, B). The β-secretase inhibitor C3 (30) and the γ-secretase inhibitor DAPT had no effect (Fig. 3A, B). A combined treatment with TAPI-1 and GI254023X did not further decrease sTACI production (Fig. 3A), suggesting that besides ADAM10 no other protease susceptible to inhibition by TAPI-1 is involved in TACI cleavage. TAPI-1 also reduced the release of sTACI from primary human B cells activated with CD40L and IL-21, while DAP Thad no effect (Fig. S1C). To confirm the role of ADAM10 in TACI cleavage, we lentivirally transduced Raji cells with shRNAs targeting ADAM9, ADAM10, ADAM17 and ADAM19 (Fig. 3C, D). As knock-down of ADAM17 influenced cell survival and proliferation, we corrected sTACI levels for cell number. Knock-down was confirmed by quantitative PCR (Fig. 3E). We found that only a knock-down of ADAM10 lead to a decrease of sTACI production (Fig. 3D), paralleled by an increase of TACI surface expression (Fig. 3C). None of the other applied shRNAs influenced membrane-bound TACI or sTACI production (Fig. 3C, D). The effect mediated by ADAM10 shRNA was similar to that of TAPI-1 in ELISA and FACS (Fig. 3A, B). To confirm the role of ADAM10 in TACI shedding in primary B cells, we used murine B cells from conditional ADAM10 KO mice which contain two flox sites flanking the ADAM10 gene(26). To achieve the ADAM10 knock-out, B cells were transduced with retroviral particles expressing the CRE-recombinase followed by the self-cleaving T2A peptide (31)and GFP (CRE-T2A-GFP), which resulted in efficient knock-out of ADAM10 as confirmed by Western Blot (Fig. S2A). As a control a retrovirus expressing GFP was used. TACI expression was determined by FACS. Knock-out of ADAM10 led to an increase of TACI surface expression when comparing cells transduced with the control virus to cells transduced with the CRE-T2A-GFP virus (Fig. S2B) and also when GFP positive cells where compared to GFP negative cells after transduction with the CRE-T2A-GFP virus (Fig. S2C, D, E). This shows that ADAM10 also sheds TACI from primary murine B cells.

γ -secretase cleaves the CTF of TACI

As ectodomain shedding of type-I membrane proteins is commonly followed by γ-secretase cleavage of the CTF (25), we investigated whether γ-secretase is also involved in the cleavage of the TACI CTF. When we transfected TACI with a C-terminal FLAG-tag in HEK293T cells and treated them with DAPT, we observed accumulation of the TACI CTF (Fig. 4A), which demonstrates that the CTF of TACI is cleaved by γ-secretase. As a next step we investigated potential functional consequences of TACI-cleavage by γ-secretase. We used an NFκB-reporter assay in HEK293T cells transfected with either full-length human TACI (TACI-FL) or TACI lacking the ectodomain (TACI-Δ-Ecto) resembling the TACI CTF. Both TACI-FL and TACI-Δ-Ecto exhibited constitutive NFκB-activation in the absence of a ligand (Fig. 4B). Spontaneous dimerization via the CRD1 (12)is believed to confer constitutive activity to TACI. TACI-Δ-Ecto lacks the CRD1. The CTF might therefore dimerizeindependent of the CRD1 due to high amounts of TACI present in this over expression system. Additionally we stimulated the transfected cells with BAFF. As expected, BAFF increased NFκB-activation in cells transfected with TACI-FL, but did not

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activate TACI-Δ-Ecto, which lacks the ligand binding domain (Fig. 4B). DAPT treatment increased NFκB-signaling mediated by TACI-Δ-Ecto indicating that cleavage of the CTF of TACI by γ-secretase reduces NFκB-activation. This suggests that γ-secretase cleavage of TACI CTF might be relevant to limit NFκB-activation after TACI ectodomain shedding. DAPT treatment had no effect on cells transfected with TACI-FL presumably because the amount of TACI cleaved by ADAM10 compared to TACI-FL was too low.

sTACI assembles in homotypic interaction

To establish whether sTACI exists as a monomeric or an oligomeric form we co expressed TACI variants with an N-terminal HA-tag or FLAG-tag in HEK293T cells. We found that sTACI-N-HA could be co-immunoprecipitated with sTACI-N-FLAG, which argues for a homotypic interaction of sTACI monomers in the absence of ligand (Fig. 5A). As a positive control, full-length TACI-N-HA derived from cell lysates was co-immunoprecipitated with full-length TACI-N-FLAG, since membrane-bound TACI was reported previously to interact homotypically(12) (Fig. 5B).

sTACI binds BAFF and APRIL, blocking B-cell survival and NFκB-activation

To explore the function of sTACI generated after ADAM10 cleavage, we determined binding of sTACI to BAFF and APRIL. We established an ELISA where sTACI was captured by BAFF-FLAG or APRIL-FLAG immobilized on an anti-FLAG coated ELISA-plate. We found that sTACI as well as TACI-Fc bound both BAFF and APRIL(Fig. 6A). In the following, we evaluated the functional consequences of this binding and determined the influence of sTACI on BCMA mediated NFκB-activation in the presence of BAFF and APRIL in HEK293T cells. In our assay, transfection of BCMA leads to lower constitutive activity and higher activation after stimulation compared to TACI transfection and was therefore more suitable for testing a possible decoy function of sTACI. Addition of sTACI to APRIL and BAFF-stimulated cells, dose-dependently blocked NFκB-activation (Fig. 6B). BAFF-mediated NFκB-activation could be blocked slightly more efficiently (Fig. 6B). Recombinant TACI-Fc was used as a control and exhibited comparable blocking capacity as sTACI (Fig. 6B). To further support the functional relevance of sTACI we tested the impact of sTACI on survival of primary B cells. We purified mouse B cells from spleens, stimulated them with anti-IgM plus BAFF or APRIL and increasing concentrations of sTACI. BAFF increased B-cell survival more strongly than APRIL. This enhanced survival by both ligands could efficiently be blocked by sTACI and TACI-Fc (Fig. 6C). These data point to a negative regulatory function of sTACI on B-cell survival.

sTACI as a potential biomarker in human immunopathologies

We evaluated whether sTACI is detectable in: (a) inflammatory CNS diseases with compartmentalized IgG production (MS, neuroborreliosis (NB)) and (b) a systemic autoimmune disease (SLE). We found significantly increased sTACI levels in the cerebrospinal fluid (CSF) of MS patients compared to patients with other neurological diseases (ONDs) without signs of CNS inflammation (Fig. 7A). Moreover, sTACI levels correlated strongly with intrathecal IgG-production (Fig. 7B). This correlation was confirmed in a second cohort of 25 MS patients ($p<0.0001$, $r=0.82$).We investigated whether this elevation is specific for MS or a consequence of compartmentalized inflammation in the

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brain. Therefore, we analysed patients with NB, which is like MS characterized by intrathecal IgG production. We detected elevation of sTACI and correlation to intrathecal IgG production also in NB, which suggests that increased sTACI levels in the CSF are not disease-specific, but the consequence of local accumulation and activation of B cells. In contrast to CSF, in plasma no significant difference between patients with MS and ONDs was detected (Fig. S3A).sTACI levels in CSF did not differ between clinically isolated syndrome (CIS), relapsing-remitting MS (RR-MS) and secondary progressive MS (SP-MS)patients (Fig. S3B). Immunosuppressive treatment influenced sTACI levels as we found decreased sTACI levels in the CSF of MS patients 12 months after initiation of monthly intravenous natalizumab treatment (Fig. 7C). Furthermore, sTACI levels were transiently reduced three days after corticosteroid treatment and returned to baseline levels within a period of 4 weeks (Fig. S3C). In SLE, we found significantly increased sTACI levels in untreated patients compared to healthy control patients (Fig. 7D) which decreased upon treatment (Fig. 7D). Interestingly, sTACI levels in untreated patients correlated with disease activity as expressed by the SLE Disease Activity Index (SLEDAI) (Fig. 7E).

Discussion

Here we report that the membrane-bound BAFF-APRIL-receptor TACI undergoes ectodomain shedding by ADAM10 and consecutive cleavage by γ-secretase. Shedding of TACI was a consequence of membrane expression of TACI on activated B cells. The generation of soluble receptors is a common principle involving a variety of membrane proteins like growth factors, receptors and their ligands, cytokines, and cell adhesion molecules (20, 21).We identified ADAM10 as the sheddase releasing sTACI while the prototype of this super family, TNFR1, is shed by ADAM17(32). ADAM10 is believed to be mainly involved in the constitutive shedding of membrane proteins, whereas ADAM17 can be activated by PMA(33). Consistent with this principle, we found ADAM10-dependent sTACI production to mainly reflect TACI expression on the cell surface and observed only little induction of shedding by PMA.

TACI shedding was followed by γ-secretase cleavage of the remaining membrane stub representing the CTF. It is unclear whether cleavage of TACI by γ-secretase solely leads to the degradation of the TACI CTF (proteasome of the membrane) (34), or to the generation of a TACI intracellular domain with signaling function like it is the case for a few other described γ-secretase substrates (e.g. Notch (35)). Inhibition of γ-secretase increased ligand-independent NFκB-activation mediated by TACI-Δ-Ecto in transfected HEK293T cells. We speculate that cleavage of the TACI CTF by γ-secretase also takes place in primary B cells thereby restricting TACI-mediated NFκB-activation after shedding of the ectodomain. However formal proof for this hypothesis is lacking at the moment.

Previous work with TACI−/− mice and CVID patients revealed a dual role of TACI in B-cell homeostasis (15–19). On the one hand, TACI promotes IgG and IgA class switch recombination(18, 36), maintains antibody production (18, 19), and increases survival of malignant B cells (37). On the other hand, TACI has negative regulatory effects on B cells, since TACI-deficient mice show a high number of hyper-reactive B cells resulting in autoimmunity and lymphoma development (15–17). Mechanisms that might contribute to

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this negative regulatory effect include (a) TACI-mediated induction of BLIMP-1, which orchestrates the switch from B-cell proliferation to plasma cell-differentiation (38), (b) involvement of TACI in activation-induced cell death of marginal zone B cells (39), and (c) the role of TACI in central removal of auto reactive B cells (40). We propose that the release of endogenous sTACI contributes to these negative regulatory features of TACI, since the decoy functions of sTACI reduce BAFF- and APRIL-mediated survival of different B-cell subpopulations.

We found sTACI to interact homotypically, analogously to membrane-bound TACI that preassembles as an oligomeric complex prior to ligand binding (12). Oligomerization increases the binding avidity for ligands, so that the oligomeric structure of sTACI suggests a functionally relevant decoy activity. The fusion protein TACI-Fc (atacicept), which is used in clinical trials, is dimerized via its Fc-domain (41). We compared endogenous sTACI with the pharmacological agent TACI-Fc in ELISAs analysingthe binding of BAFF and APRIL, in NFκB-reporter assays and in survival assays with primary B cells. These experiments showed that endogenous sTACI and TACI-Fc share essential decoy functions. TACI-Fc (atacicept), reduced immunoglobulin levels and mature B-cell counts in clinical studies(9). All this suggests that endogenous sTACI is a similar negative regulator of the B-cell compartment in vivo. We propose that an equilibrium of sTACI and BAFF controls B-cell numbers fitting to the observation of raised BAFF levels and increased survival of naïve B cells in TACI−/− mice(15, 16).

We analysed sTACI levels in patients with systemic and compartmentalized systemic autoimmune diseases. Our data indicate that sTACI is produced by locally or systemically accumulating activated B cells and plasma cells. In SLE, sTACI was elevated in serum. This can readily be explained by a hyper-activation of the B-cell compartment and increased levels of circulating plasma cells in SLE(3, 42). Interestingly, in SLE we observed a close correlation of sTACI levels with disease activity. This suggests that sTACI might serve as a useful biomarker, e.g., for individualizing (and thereby optimizing) B-cell targeting therapies. In a phase II clinical trial investigating the effects of belimumab in SLE patients, patients with high concentrations of ANA and anti-dsDNA antibodies responded better to treatment (43). However, baseline BAFF values did not correlate with treatment response (44). Whether sTACI helps to identify patients who are likely to benefit from belimumab or other B-cell directed therapies remains to be studied. In MS, sTACI was not increased in blood, but elevated in CSF, where levels correlated with intrathecal Ig-production. Natalizumab treatment decreased sTACI levels in the CSF fitting to the concept that natalizumab prevents entry of VLA-4^+ lymphocytes into the CNS, including circulating B cells that express substantial levels of VLA-4 (45). The fact that atacicept increased disease activity in MS patients(9), whereas in SLE it showed beneficial clinical effects (10), remains incompletely understood. Expression of BAFF-R on neurons was observed(46). So one could speculate that atacicept could limit neuronal survival by decreasing BAFF-levels. Furthermore, BAFF was shown to induce IL-10 producing regulatory B cells that are able to inhibit T cell proliferation. This induction was blocked by TACI-Fc(10). Therefore, TACI-Fc could interfere in the equilibrium between sTACI and BAFF involved in fine-tuning the balance between effector B cells and regulatory B cells.

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In summary, the discovery of the existence of sTACI and its effects as a negative immunoregulator extends our understanding of the complexity of the BAFF-APRIL system. Our study unravels the biochemical basis of sTACI generation. Further, we show that sTACI offers potential as a novel biomarker in MS and SLE, and may be useful for therapy optimization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ADAM	A disintegrin and metalloproteinase
APRIL	a proliferation-inducing ligand
BAFF	B cell-activating factor
CIS	clinical isolated syndrome
CSF	cerebrospinal fluid
HC	healthy control
MS	multiple sclerosis
NB	neuroborreliosis
OND	other neurological diseases
RIP	regulated intramembrane proteolysis
RR-MS	relapsing-remitting MS
SP-MS	secondary progressive MS
sTACI	soluble TACI
TACI	transmembrane activator and CAML interactor.

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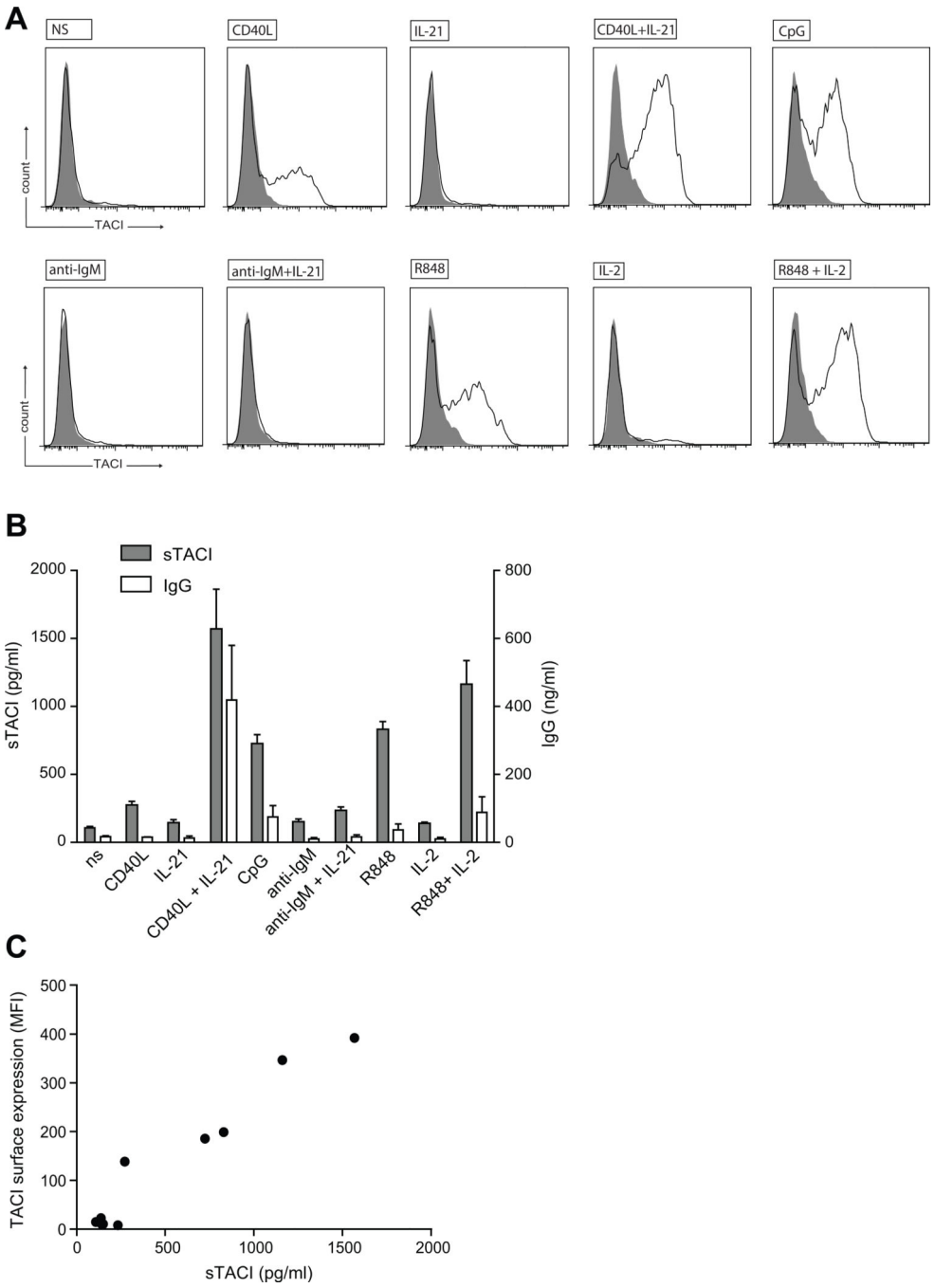

Figure 1. sTACI is released by activated B cells and closely correlates with membrane-bound TACI

(**A, B**) Human purified B cells were activated using CD40-L, IL-21, CD40L + IL-21, CpG (ODN2006), anti-IgM, anti-IgM + IL-21, R848, IL-2 and R848 + IL-2 for 4 days. (**A**) TACI surface expression was determined by FACS 4 days after activation. Filled histograms represent the isotype control, solid lines the TACI expression. (**B**) sTACI and IgG production were analysed by ELISA. (**C**) Mean fluorescence intensity (MFI) of TACI surface expression was calculated by subtracting the isotype fluorescence signal. sTACI

production and MFI of membrane bound TACI correlated strongly (p=0.0105, r=0.7818, Spearman correlation). Representative data of two different donors.

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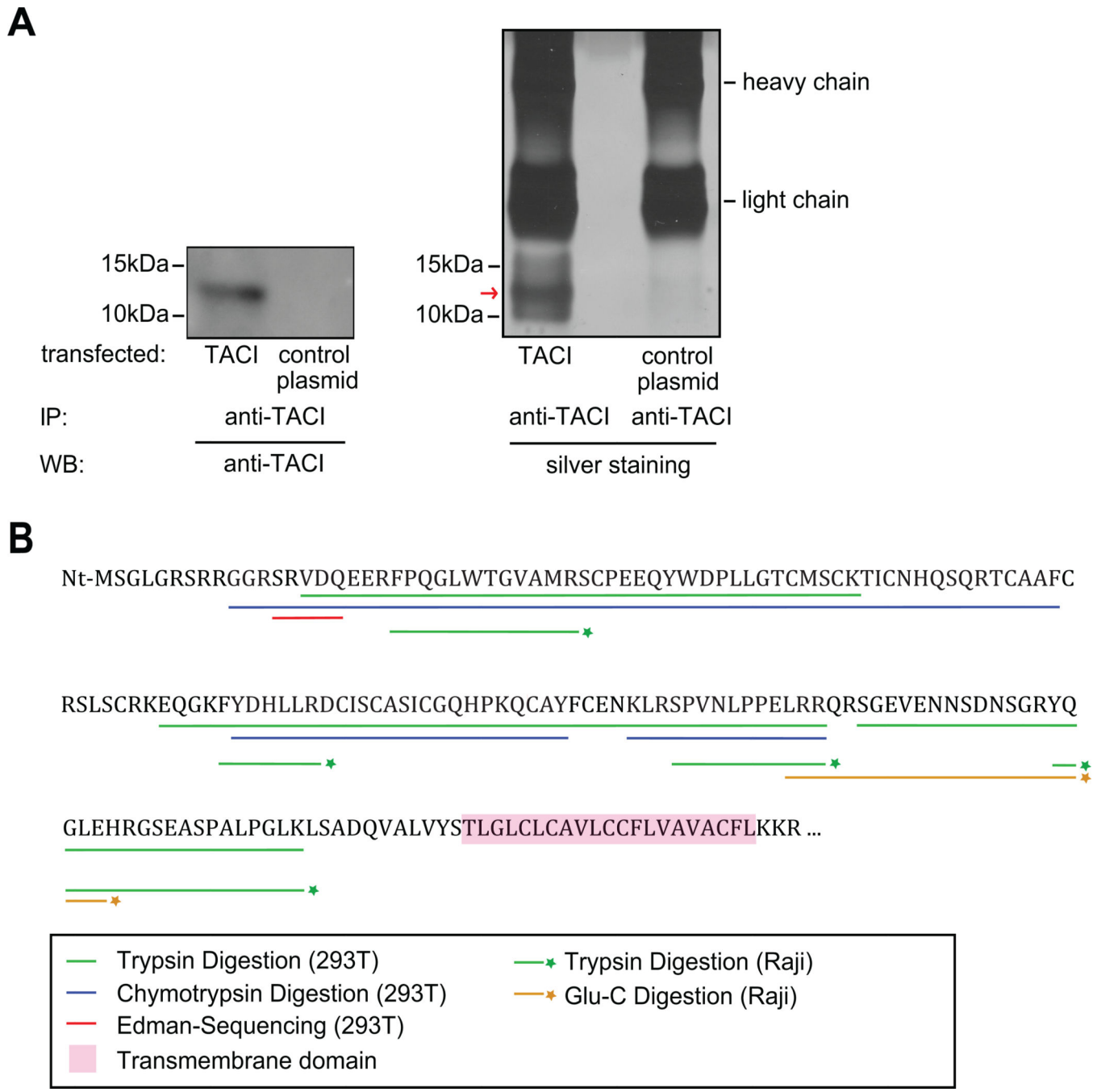

Figure 2. sTACI is composed of the extracellular part of TACI

(**A**) sTACI was immunoprecipitated from supernatant of HEK293T cells transfected with full-length TACI or an empty vector as control (IP: MAB174) followed by SDS-PAGE and Western blotting (WB). IP eluates were probed for TACI (MAB174). Silver staining was performed after IP from supernatant of HEK293T cells transfected with full-length TACI or an empty vector as control(IP: AF174). (**B**) After IP of sTACI from HEK293T cell supernatant the band corresponding to sTACI in silver staining was excised and digested with trypsin or chymotrypsin. IP of sTACI from Raji cell supernatant followed by acidic

elution and digestion with trypsin or GluC was performed. The aa sequences of TACI and peptides identified by mass spectrometry after tryptic (green), chymotryptic (blue) or GluC (orange) digestion are shown. sTACI derived from HEK293T cells was additionally analysed by N-terminal-sequencing (Edman) (red).

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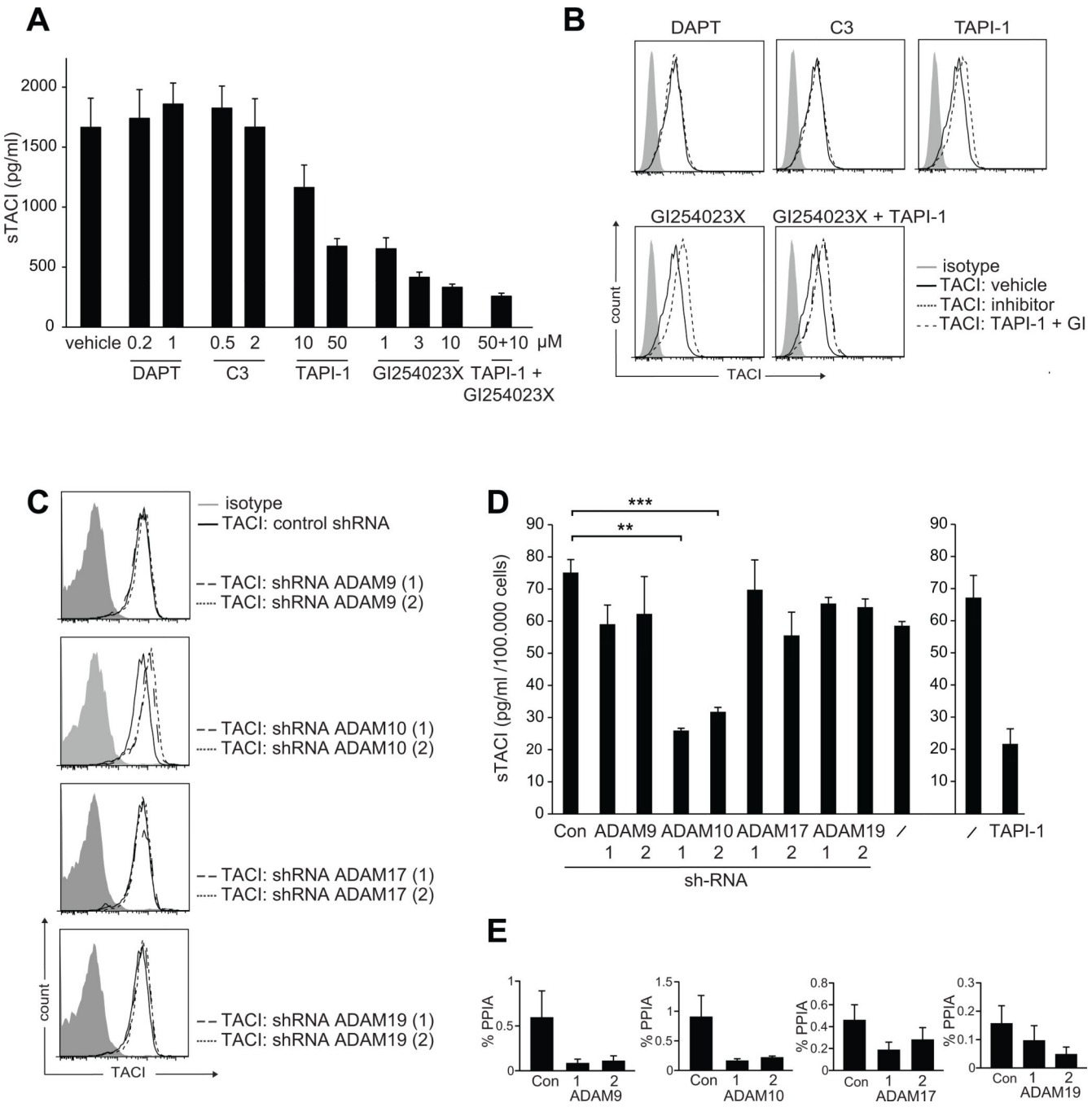

Figure 3. ADAM10 sheds TACI

(**A, B**) Raji cells were treated for 24 hours with the indicated concentrations of DAPT, C3, TAPI-1, GI354023X and TAPI-1 + GI354023X. (**A**) sTACI release was determined by ELISA (combined data of 4 independent experiments (mean ± SEM)). (**B**) TACI surface expression was analysed by FACS. Only the high inhibitor concentrations with corresponding vehicle control and isotype controls are depicted (representative data of 4 independent experiments). (**C, D, E**) Raji cells were lentivirally transduced with two different shRNAs targeting ADAM9, ADAM10, ADAM17, ADAM19 respectively or a

non-targeting shRNA (Con). For comparison cells were treated with TAPI-1. (**C**) TACI surface expression was analysed by FACS; representative data of 2 independent experiments. (**D**) sTACI release was determined by ELISA and controlled for cell number (two-tailed, paired T-test, including 2 technical replicates of the respective experiments); combined data of 2 independent experiments (mean ± SEM). (**E**) Knock-down of ADAM9, ADAM10, ADAM17 and ADAM19 was determined by qPCR; 1 and 2 represent two different shRNAs targeting the same ADAM; combined data of two independent experiments (mean ± SEM).

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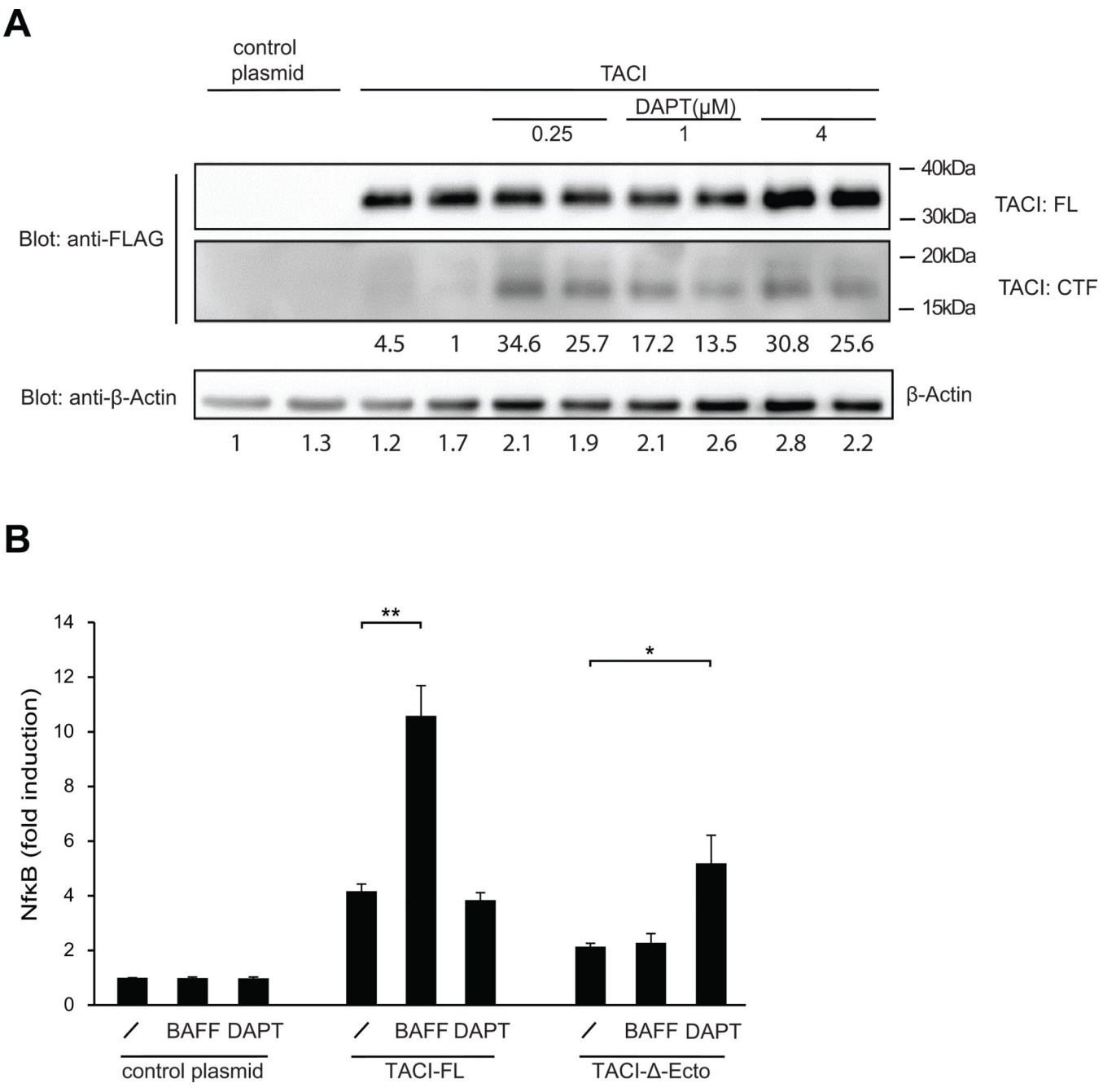

Figure 4. γ-secretase degrades the CTF of TACI shutting down NFκB-activation

(**A**) HEK293T cells were transfected with full-length TACI with a C-terminal FLAG-tag and treated with the indicated concentrations of DAPT or vehicle control. After 48 hours lysates were obtained for SDS-PAGE and WB. Lysates were probed for expression of tagged TACI demonstrating full-length (FL) TACI and the CTF of TACI (representative data of 3 independent experiments). Relative intensities are depicted below the bands. (**B**) HEK293T cells were transfected with 1 ng of full-length TACI (TACI-FL) or TACI lacking the ectodomain (TACI-Δ-Ecto) and a luciferase-based NFκB-reporter. DAPT (1 μM) and

BAFF (100 ng/ml) were added as indicated and NFκB-activation was determined (two-tailed, paired T-tests); combined data of 5 independent experiments (mean ± SEM).

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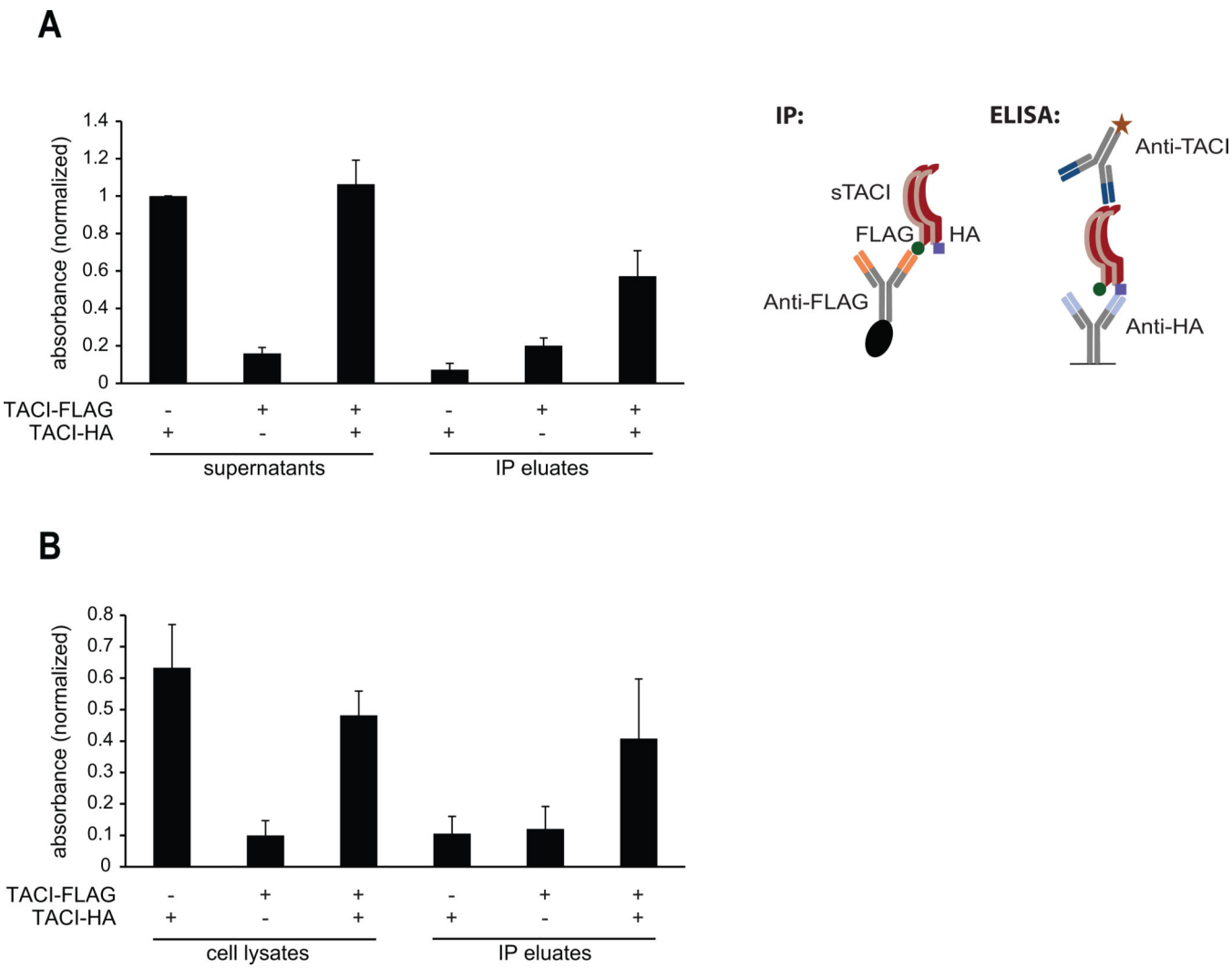

Figure 5. TACI assembles homotypically independent of a ligand

(**A, B**) HEK293T cells were transfected with expression plasmids containing TACI with an N-terminal FLAG-tag or HA-tag as indicated. 48 hours after transfection, supernatants (**A**) were harvested and cell lysates (**B**) were prepared. A part of the supernatants (**A**) and lysates (**B**) prior to IP were stored; the remaining supernatants and lysates were subjected to FLAG-IP and the IP eluates plus the supernatants and lysates stored prior to IP were measured by ELISA. An anti-HA antibody was used for coating and an anti-TACI antibody was applied for detection; combined data of 3 independent experiments (mean ± SEM).

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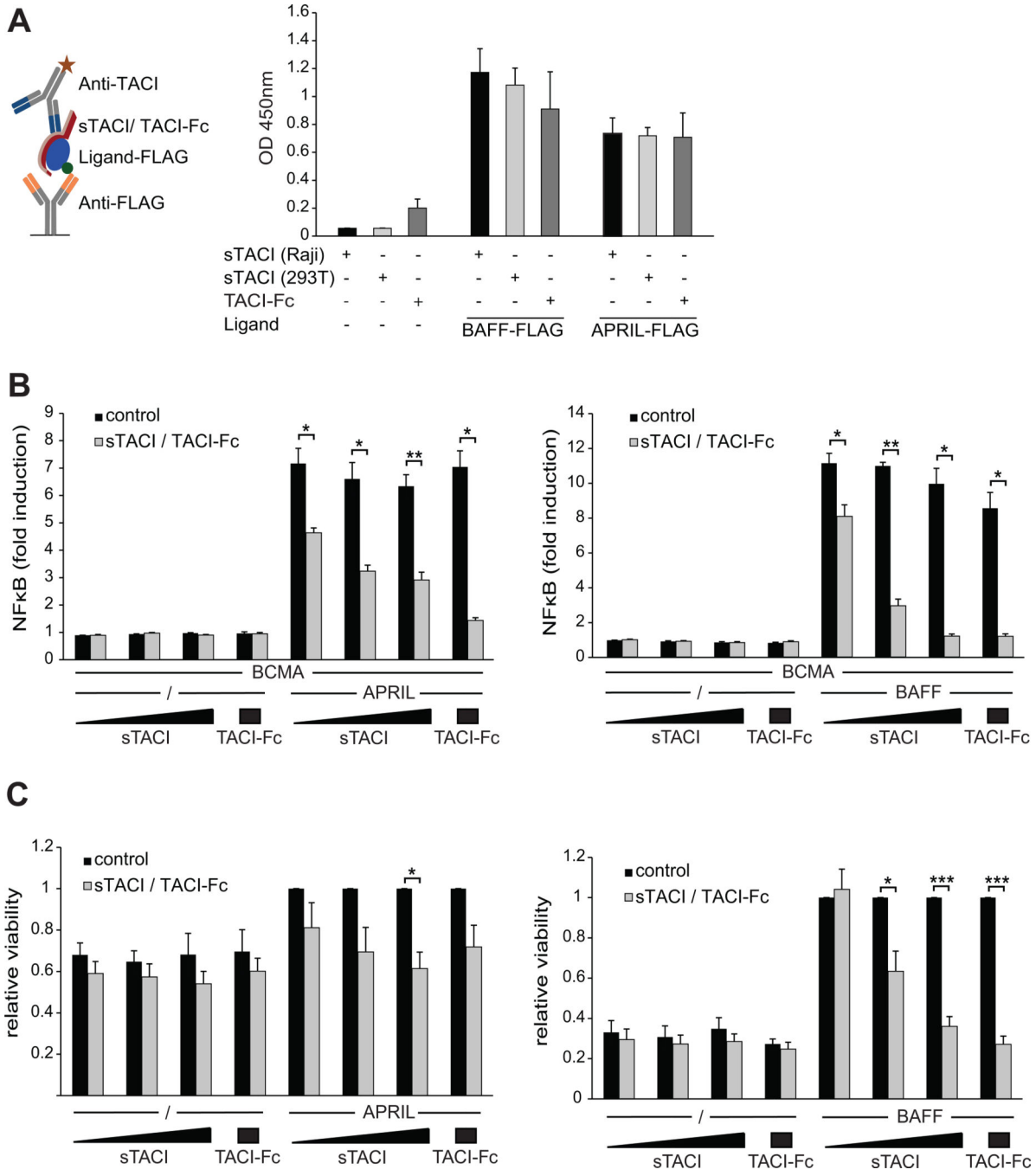

Figure 6. sTACI acts as a decoy for BAFF and APRIL and blocks B-cell survival

(**A**) ELISA plates were coated with anti-FLAG-antibodies (M2, 5μg/ml); BAFF-FLAG and APRIL-FLAG were added; sTACI (25 ng/ml) derived from supernatant of transfected HEK293T cells or concentrated supernatants of Raji cells and TACI-Fc (25 ng/ml) were used; combined data of 3 independent experiments (mean±SEM). (**B**) HEK293T cells were transfected with full-length BCMA (2.5 ng) and a luciferase-based NFκB-reporter. BAFF and APRIL (100 ng/ml) were added together with increasing amounts of sTACI containing supernatant (33, 100 and 300 ng/ml), control supernatant or TACI-Fc (300 ng/ml) and

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NFκB-activation was determined (two-tailed, paired T-test); combined data of 3 independent experiments (mean ± SEM). (**C**) Murine B cells were activated via anti-IgM and cultured for 2 days with APRIL or BAFF (100 ng/ml) in the presence or absence of sTACI (100, 200 and 400 ng/ml) and TACI-Fc (400 ng/ml). Survival of B cells was determined by FACS analysing the percentage of living $CD19^+$ cells after staining with TO-PRO®-3 Iodide. Viability was calculated in relation to the BAFF- and APRIL- induced survival that was assigned as 1 (two-tailed, paired T-test between BAFF treated and BAFF + sTACI/ TACI-Fc treated conditions); combined data of 4–5independent experiments (mean ± SEM).

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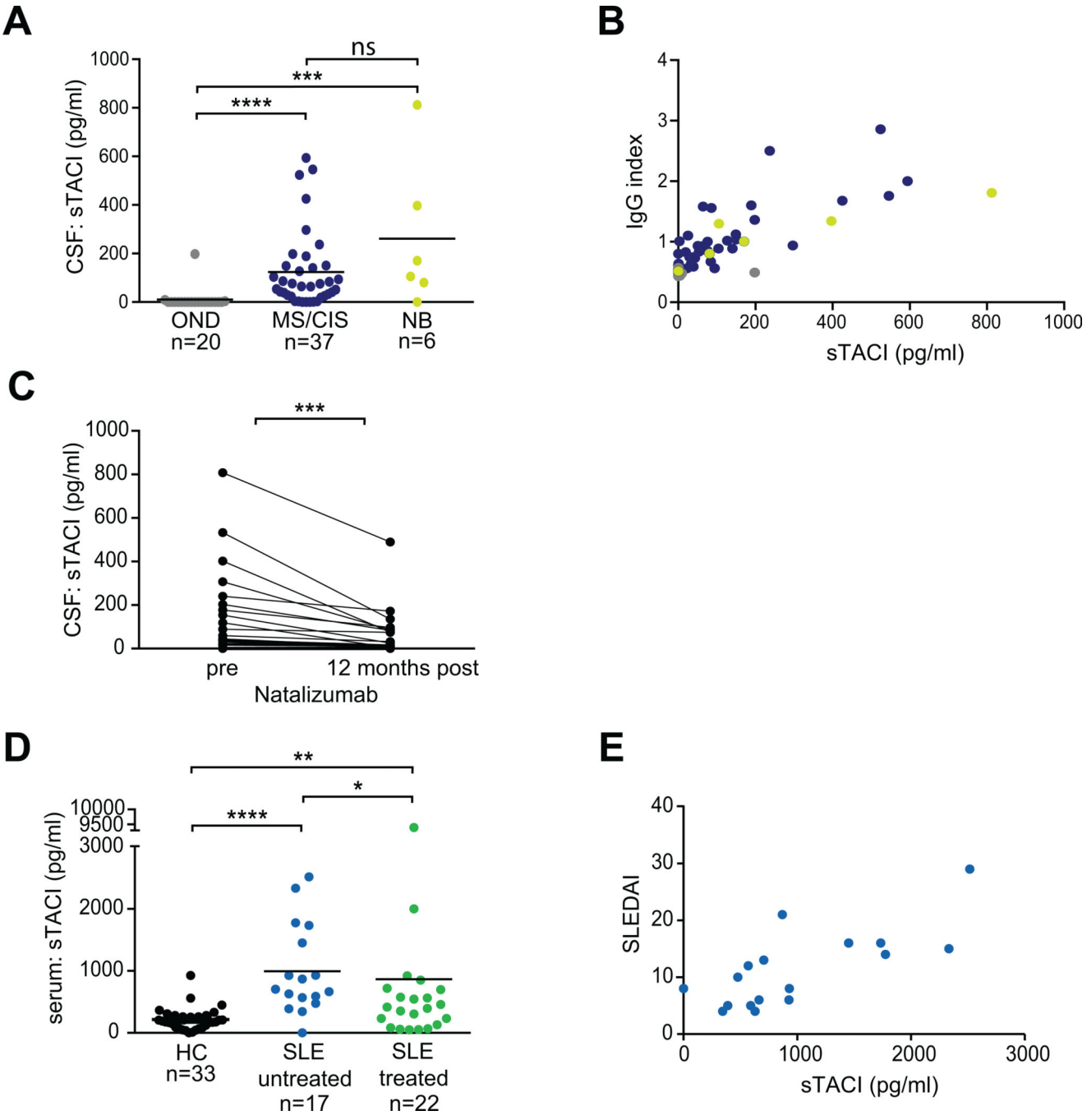

Figure 7. sTACI as a biomarker

(**A, B, C, D, E**) sTACI levels were determined by ELISA in CSF or serum; horizontal bars indicate the mean. (**A**) sTACI is elevated in the CSF of patients with CIS/MS and NB compared to ONDs (two-tailed, unpaired and non-parametric T-test). (**B**) sTACI in the CSF correlates with intrathecal IgG production (MS/CIS: $p < 0.0001$, $r = 0.75$, NB: $p = 0.0167$, $r = 0.9429$, OND: ns, Spearman correlation); some data points of OND patients are hidden behind data points of CIS/MS and NB patients. (**C**) sTACI levels in CSF decrease after 12 months of natalizumab treatment (n=25) (two-tailed, paired T-test). (**D**) sTACI is elevated in

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the serum of untreated SLE patients and less prominent also in the treated SLE cohort compared to HC (two-tailed, unpaired and non-parametric T-test); with treatment sTACI levels decrease compared to the untreated SLE patients (two-tailed, unpaired and non-parametric T-test). (**E**) sTACI in serum of untreated SLE patients correlates strongly with disease activity quantified by the SLEDAI ($p=0.0019$; $r=0.697$, Spearman correlation).