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Interactions of Tumor Necrosis Factor (TNF) and TNF Receptor Family Members in the Mouse and Human*

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Ligands of the tumor necrosis factor superfamily (TNFSF) (4–1BBL, APRIL, BAFF, CD27L, CD30L, CD40L, EDA1, EDA2, FasL, GITRL, LIGHT, lymphotoxin α, lymphotoxin αβ, OX40L, RANKL, TL1A, TNF, TWEAK, and TRAIL) bind members of the TNF receptor superfamily (TNFRSF). A comprehensive survey of ligand-receptor interactions was performed using a flow cytometry-based assay. All ligands engaged between one and five receptors, whereas most receptors only bound one to three ligands. The receptors DR6, RELT, TROY, NGFR, and mouse TNFRH3 did not interact with any of the known TNFSF ligands, suggesting that they either bind other types of ligands, function in a ligand-independent manner, or bind ligands that remain to be identified. The study revealed that ligand-receptor pairs are either cross-reactive between human and mouse (*e.g.* Tweak/Fn14, RANK/RANKL), strictly species-specific (GITR/GITRL), or partially species-specific (*e.g.* OX40/OX40L, CD40/CD40L). Interestingly, the receptor binding patterns of lymphotoxin α and αβ are redundant in the human but not in the mouse system. Ligand oligomerization allowed detection of weak interactions, such as that of human TNF with mouse TNFR2. In addition, mouse APRIL exists as two different splice variants differing by a single amino acid. Although human APRIL does not interact with BAFF-R, the shorter variant of mouse APRIL exhibits weak but detectable binding to mouse BAFF-R.

Although the tumor necrosis factor (TNF)[5] superfamily (TNFSF) does not exist in prokaryotes, yeast, or nematodes, it is represented by a single member in insects and has significantly expanded in vertebrates through gene duplication events, with 18 genes identified so far in mouse and human (1). In *Drosophila*, the single TNF homologue Eiger induces c-Jun N-terminal kinase-mediated cell death and has been shown to modulate host response to *Salmonella* infection (2–4). EDA, the closest vertebrate homologue of Eiger, is required for the development of scales in fishes, feathers in birds, and hair, teeth, and sweat glands in mammals (5). In birds and mammals, however, most TNFSF and TNFRSF members are implicated in the development, maintenance, and function of immune cells and secondary lymphoid organs and participate in other functions, such as bone homeostasis (1). Several TNFSF and TNFRSF members are being used or evaluated as drug targets for the treatment of immune dysfunctions, cancer, and other diseases (for reviews, see Refs. 6–10).

TNF family ligands are type II transmembrane proteins with an extracellular, homotrimeric C-terminal TNF homology domain that is frequently released as a soluble cytokine upon proteolytic processing (11). Lymphotoxin β is remarkable in that it cannot form homotrimers but instead heterotrimerizes with lymphotoxin α (12). Receptors are usually type I and sometimes type III (BCMA, TACI, BAFF-R, and XEDAR) membrane proteins, with the exception of OPG and DcR3, which are secreted. Receptors are characterized by the presence of one to four cysteine-rich domains (CRD) in their extracellular portion. Those receptors with several CRDs adopt an elongated structure and bind at the interface between two ligand monomers within a trimer, whereas single CRD receptors are more compact and contact a single ligand monomer in a trimeric ligand (13–16). Generally, one trimeric ligand engages three monomeric receptors, a key event for the activation of intracellular signaling pathways.

The TNF ligands have been shown to bind to one, two, or more different receptors and vice versa. For example, RANKL promotes bone resorption by stimulating the maturation of osteoclast precursors through the membrane-bound receptor RANK and is regulated through binding to the soluble decoy receptor OPG (10, 17). Experimental data regarding TNFSF-TNFRSF interactions in human and mouse and more specifically those addressing interspecies cross-reactivities are scattered in the literature (if available at all) and rely on different experimental settings or on specific reagents. In this study, we have conducted a systematic survey of TNFSF-TNFRSF interactions and report reactivities and cross-reactivities of human and mouse proteins.

MATERIALS AND METHODS

Cell Lines and Reagents—HEK293T cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 5 μg/ml each penicillin and streptomycin. Heparin (Liquemin 5000 IU/ml) was purchased from Roche Applied Science. The rat IgG2a monoclonal antibody anti-TRAILR3 (LEIA) was purchased from

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[5] The abbreviations used are: TNF, tumor necrosis factor; TNFSF, TNF superfamily; TNFRSF, TNF receptor superfamily; BAFF, B cell-activating factor of the TNF family; BCMA, B cell maturation antigen; TACI, transmembrane activator and calcium modulator and cyclophilin ligand interactor; APRIL, a proliferation-inducing ligand; EDA, ectodysplasin A; EDAR, EDA receptor; XEDAR, X-linked EDAR; GITR, glucocorticoid-induced TNF receptor; GITRL, GITR ligand; HVEM, herpes virus entry mediator; LIGHT, lymphotoxin homologue inducible competing with glycoprotein D for HVEM (a receptor expressed on T cells); LINGO-1, leucine-rich repeat and Ig domain-containing Nogo receptor-interacting protein; NogoR, Nogo receptor; LTαβ, lymphotoxin αβ; LTβR, lymphotoxin β receptor; RANK, receptor activator of NFκB; RANKL, RANK ligand; TWEAK, TNF family member with weak apoptosis-inducing activity; DcR3, decoy receptor 3; DR, death receptor; Fn14, product of fibroblast growth factor-inducible gene 14; NGF, nerve growth factor; NGFR, nerve growth factor receptor; OPG, osteoprotegerin; RELT, receptor expressed in lymphoid tissues; GPI, glycosyl-phosphatidylinositol; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; PE, phosphatidylethanolamine; TRAIL, TNF-related apoptosis-inducing ligand; CD, cluster of differentiation; TL, TNF family ligand; TNFRH3, TNF receptor homologue 3; CRD, cysteine-rich domain; h, human; m, mouse.

Axxora (San Diego, CA), the biotinylated anti-FLAG M2 antibody from Sigma, PE-coupled streptavidin from eBiosciences, and PE-coupled goat anti-human IgG and PE-coupled goat anti-rat IgG (H+L) from Southern Biotech (Birmingham, AL).

Expression Constructs—An expression vector for Fc:ligands has been described previously (18) and was used for the expression of the following ligands (amino acid numbers are given in parentheses; = means amino acid sequence is 100% identical to): h4–1BBL-(85–254), m4–1BBL-(140–310), hAPRIL-H98-(98–233), mAPRIL-H98(ΔA112)-(98–232), mAPRIL-H98(A112)-(98–233), hBAFF-(134–285), mBAFF-(127–309), hCD27L-(39–193), mCD27L-(52–195), hCD30L-(63–235), mCD30L-(71–239), hCD40L-(116–61), mCD40L-(115–260), mEDA1-(=hEDA1)-(245–391), mEDA2(=hEDA2)-(245–389), hFasL-(139–281), mFasL-(106–279), hGITRL-(47–177), mGITRL-(38–173), hLIGHT-(89–240), mLIGHT-(87–239), hLTα-(43–205), mLTα-(52–202), hLTβ-(63–244), mLTβ-(63–306), hOX40L-(50–183), mOX40L-(140–310), hRANKL(152–317), mRANKL-(157–316), hTL1A-(93–251), mTL1A-(94–252), hTNF-(85–233), mTNF-(76–235), hTRAIL-(95–281), mTRAIL-(120–291), hTWEAK-(141–284), mTWEAK-(94–249).

FLAG-tagged hLTα-(43–205) and mLTα-(52–202) were expressed with the pFLAG vector (19) and co-transfected with the corresponding Fc:LTβ to produce mouse and human LT$\alpha\beta$. Although we did not determine the ratio of LT$\alpha_1\beta_2$ and LT$\alpha_2\beta_1$ in these preparations, they probably contained a mixture of both. hTNF-(85–233), mTNF-(76–235), mBAFF-(127–309), mAPRIL(ΔA112)-(98–232), and mAPRIL(A112)-(98–233) were also cloned into the pFLAG vector.

An expression vector for the extracellular domains of receptors fused to a portion of hTRAILR3 (157–259), including the GPI addition signal, has been described previously (20) and was used to clone the indicated sequence of various receptors that either contained their own signal peptide or, when indicated, were cloned after the hemagglutinin or immunoglobulin G1 heavy chain signal peptides (HA signal and Ig signal, respectively): h4–1BB-(1–186), m4–1BB-(1–181), hBAFF-R-(2–71) (HA signal), mBAFF-R-(2–70) (HA signal), hBCMA-(2–54) (Ig signal), mBCMA-(1–46) (Ig signal), hCD27-(1–191), mCD27-(1–182), hCD30-(1–380), mCD30-(1–285), hCD40-(1–193), mCD40-(1–193), hDcR3-(1–300), hDR3-(25–199) (Ig signal), mDR3-(1–194), hDR6-(1–351), mDR6-(1–350), mDcTRAILR1-(1–158), mDcTRAILR2-(40–171) (HA signal), hEDAR-(1–183), mEDAR-(1–183), hFas-(1–170), mFas-(1–169), hFn14-(1–75), mFn14-(1–75), hGITR-(26–161) (HA signal), mGITR-(1–153), hHVEM-(1–200), mHVEM-(1–206), hLTβR-(1–220), mLTβR-(1–217), hNGFR-(1–250), mNGFR-(1–243), hOPG-(1–202), mOPG-(1–214), hOX40-(1–214), mOX40-(1–209), hRANK-(29–213) (HA signal), mRANK-(1–200), hRELT-(1–125), mRELT-(1–165), hTACI-(2–160) (HA signal), mTACI-(2–78) (HA signal), hTNFR1-(1–211), mTNFR1-(1–210), hTNFR2-(1–257), mTNFR2 Thr-102/Ile-108-(1–257), mTNFR2 Ser-102/Thr-108-(1–257), mTNFRH3-(1–162), hTRAIL-R1-(1–239), hTRAIL-R2-(1–212), mTRAIL-R-2-(1–166), hTRAIL-R3-(1–259) (full-length), hTRAIL-R4-(1–211), hTROY-(1–168), mTROY-(1–168), hXEDAR-(1–134) (Ig signal), and mXEDAR-(2–133) (HA signal). An expression vector for Receptor:Fc has been described previously (21) and was used for the expression of hCD27-(1–191), mCD27-(1–182), mDcTRAILR1-(1–158), mDcTRAILR2-(1–171), hFas-(1–170), mFas-(1–169), hHVEM-(1–200), mHVEM-(1–206), hLTβR-(1–220), mLTβR-(1–217), hOPG-(1–202), mOPG-(1–214), mTNFRH3-(1–162), hTRAIL-R1-(1–239), hTRAIL-R2-(1–212), hTRAIL-R3-(1–240), and hTRAIL-R4-(1–211). Full-length hFasL-(1–281), mFasL-(1–279), hTRAIL-(1–281) (FLAG), mTRAIL-(1–291) (FLAG), hCD27L-(1–193) (FLAG), mCD27L-(1–195), hLIGHT-(1–240), and mLIGHT-(1–239) were cloned in the PCR3 mammalian expression vector (Invitrogen).

Transfection—For secreted proteins, 293T cells transiently transfected using the calcium phosphate procedure were grown in serum-free Opti-MEM I medium for 4–7 days. Supernatants were collected and frozen until use. In one instance (see Fig. 2*D*), supernatants were concentrated 60× before use. When concentration is indicated, protein concentration was estimated by immunoblot using anti-FLAG or anti-Fc antibodies with purified proteins of known concentration as standards.

For flow cytometry stainings, 293T cells were co-transfected overnight with expression constructs for the receptor of interest and EGFP, washed and cultured for an additional 24 h in complete medium. For staining and analysis, cells were detached by pipetting.

Flow Cytometry Staining—Stainings ($\sim 2 \times 10^5$ transfected 293T cells) were performed in round-bottomed 96-well plates for 20 min on ice with either (*a*) Fc-tagged ligands followed by PE-coupled goat anti-human IgG, (*b*) Fc-tagged receptor followed by PE-coupled goat anti-human IgG, (*c*) 0.5 μg of LEIA antibody followed by PE-coupled goat anti-rat IgG, or (*d*) FLAG-tagged ligands followed by biotinylated anti-FLAG M2 antibody and PE-coupled streptavidin. Stainings were performed with 5–15 μl (~10–50 ng) of recombinant proteins in Opti-MEM supernatants in a final volume of 25 μl of phosphate-buffered saline and 5% fetal calf serum. Heparin (0.1 μl) was added in all stainings to prevent unspecific binding of recombinant proteins to glycosaminoglycans (20). The cells were analyzed on a FACScan (BD Biosciences) using the CellQuest program.

RESULTS AND DISCUSSION

GPI-anchored TNF Receptors Bind Cognate Recombinant Fc:Ligands Fusion Proteins at the Cell Surface—We have developed an assay to monitor TNFSF-TNFRSF interactions, which requires no antibodies specific for the ligands and receptors of interest, no protein purification, and which contains an internal negative control. In this assay, the extracellular domain of each receptor is expressed as a fusion protein with the C-terminal portion of TRAIL-R3 in 293T cells, together with EGFP. The C-terminal portion of TRAIL-R3 contains a repetitive amino acid sequence and a GPI anchor that targets the receptor to the plasma membrane (22, 23). The C-terminal portion of TRAIL-R3 is specifically recognized by a monoclonal antibody (LEIA) that allows monitoring of surface expression of the chimeric receptors. Fusing receptors to a glycolipid anchor circumvents problems that could be associated with the use of full-length receptors, namely induction of apoptosis, intracellular retention, or, in the case of OPG and DcR3, secretion. Finally, receptor-expressing cells co-express EGFP, whereas untransfected, EGFP-negative cells serve as internal negative controls.

Ligands are expressed as soluble fusion proteins comprising the Fc portion of human immunoglobulin G1 and the TNF homology domain of TNFSF members. These fusion proteins are predicted to form hexamers (*i.e.* to contain two trimeric ligands) and to display higher avidity for their receptors than a regular trimer (18). The Fc is detected with a secondary PE-labeled antibody directed against human IgG1.

Cells co-transfected with EGFP, and various receptor-GPI constructs were screened for Fc:ligands binding. Although background staining of the various ligands varied, more than a hundred specific interactions were readily identified based on the curved appearance of the scattergrams (Fig. 1). Under these experimental conditions, there were several exceptions. Fc:mLIGHT (but not Fc:hLIGHT) failed to bind its cognate receptors, despite the fact that the portions of hLIGHT and mLIGHT contained in these constructs were exactly equivalent. The same was

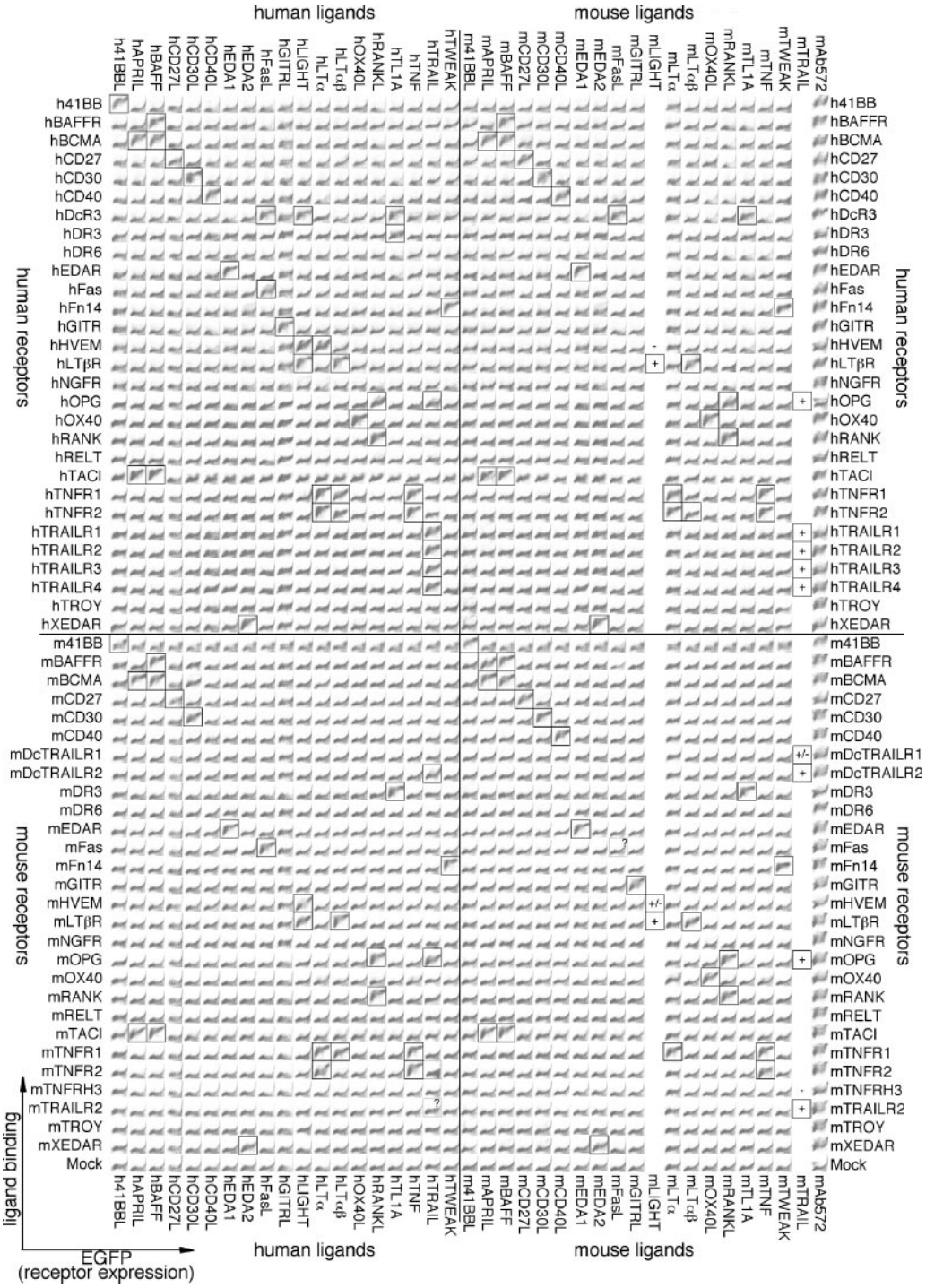

FIGURE 1. **Ligand-receptor interactions in the TNF family.** Receptors are listed on the *sides* and ligands at the *top* and *bottom* of the figure. Human proteins are designated *h*, and mouse proteins are designated *m*. Receptors fused to the GPI anchor of TRAIL-R3 were expressed in 293T cells together with an EGFP tracer (*x*-axis) and stained with ligands expressed as Fc fusion proteins (*y*-axis). Receptor expression was verified by staining with the monoclonal antibody LEIA (*mAb572*, *right column*) directed against the C-terminal portion of TRAIL-R3 that is present in all receptors. Cells were analyzed by two-color flow cytometry, and the scattergrams obtained for each receptor-ligand combination are shown. Both axes show fluorescence intensity on a logarithmic scale (10^0–10^4). Positive interactions are *boxed* and are characterized by the fact that cells transfected to a high level bind more ligand than cells with low levels of transfection. Known interactions that were not detected (hTRAIL with mDcTRAILR2, mFasL with mFas) are *boxed* in *gray dots* and contain a *question mark*. No data are shown for Fc:mLIGHT and Fc:mTRAIL, because these two ligands were not produced in an active form. +, ±, or − are shown for results obtained with full-length forms of these two ligands (see Fig. 2). As a control, cells were transfected with the empty expression vector (*Mock*). Data originate from several independent experiments performed with an identical protocol. The entire screen was performed once, but all negatives, where a positive interaction could have been reasonably expected (*e.g.* hCD40L with mCD40), and all positives were confirmed twice to many times with similar results. As the portion of EDA1 and EDA2 used in this study is identical between mouse and human, the sets of data for EDA1 and EDA2 are shown twice. The short splice variant of mAPRIL was used in these experiments (see Fig. 4).

true for Fc:mTRAIL. In addition, Fc:hTRAIL and Fc:mFasL did not recognize at least one of their described binding partners (Fig. 1 and data not shown). This could result from Cys-230 oxidation in TRAIL (25, 26), from putative detrimental effects of the Fc portion on some ligands in the Fc:ligand fusion proteins, or could be due to the portion of the ligand expressed. Fc:mFasL was expressed as two constructs, one containing

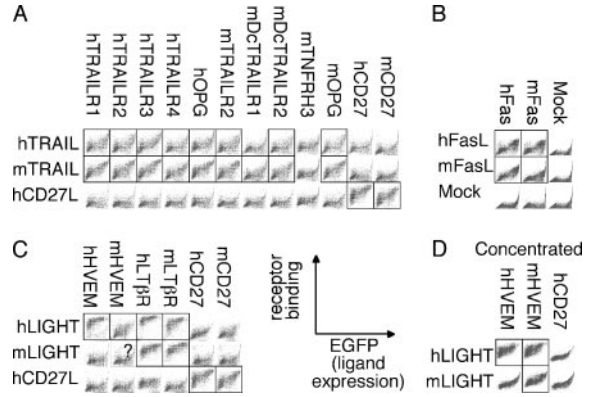

FIGURE 2. **Interactions of hTRAIL, mTRAIL, mFasL, and mLIGHT with their cognate receptors.** Full-length ligands were expressed in 293T cells together with an EGFP tracer (*x*-axis) and stained with receptor:Fc fusion proteins (*y*-axis). Both axes show fluorescence intensity on a logarithmic scale (10^0–10^4). *A*, hTRAIL, mTRAIL, and hCD27L. *B*, hFasL and mFasL. *C*, hLIGHT, mLIGHT, and hCD27L. The *question mark* denotes the absence of an expected positive interaction between mLIGHT and mHVEM. *D*, same as described for *C*, except that cell supernatants containing hHVEM:Fc, mHVEM:Fc, and hCD27:Fc were concentrated 60× before use. Similar data were obtained in two or more independent experiments.

the TNF homology domain only (data not shown) and the other containing the entire extracellular domain (Fig. 1). Only the long form demonstrated binding to hDcR3, a described receptor for hFasL (27), but neither form interacted with mFas. We therefore expressed mFasL, mLIGHT, mTRAIL, and hTRAIL as untagged, full-length proteins in 293T cells and detected interactions with recombinant receptor:Fc fusion proteins. In this format, these four ligands interacted with their cognate receptors (Fig. 2). mLIGHT interacted readily with mLTβR, but only weak binding to mHVEM was observed (Fig. 2*D*).

Simple Interactions—Some interactions involve a single ligand binding to a single receptor. This is the case for the following pairs: 4–1BBL/4–1BB, CD27L/CD27, CD30L/CD30, CD40L/CD40, EDA1/EDAR, EDA2/XEDAR, GITRL/GITR, OX40L/OX40, and TWEAK/Fn14. m4-1BBL and mGITRL did not recognize the corresponding human receptors, whereas hCD40L, hGITRL, and hOX40L did not bind the mouse receptors. In addition, h4–1BBL interacted only weakly with m4–1BB, as seen from the convex shape of the scattergram, in which only highly transfected cells bound the ligand (Fig. 1).

An interaction between TWEAK(Apo3L) and DR3 has been reported in an earlier study (28). This interaction has not been reproduced here or in a number of other settings (Fig. 1) (29–31) and should be considered an artifact. Instead, TWEAK interacts with Fn14, and DR3 binds to TL1A (Fig. 1) (32, 33).

The RANKL, TRAIL Network—Expression of soluble TRAIL in 293 cells results in the oxidation of Cys-230, which is required for the chelation of a zinc atom at the center of the trimer, and may explain the total or partial lack of activity of Fc:hTRAIL and Fc:mTRAIL (25, 26). However, the membrane-bound forms of both human and mouse TRAIL were suitable for this study of receptor binding and allowed us to reproduce the previously published interactions (Fig. 2*A*) (34).

The Immediate TNF Network—TNF, LTα, LTβ, LIGHT, TL1A, and FasL share at least one receptor with another ligand of the immediate TNF family. We have confirmed the published interactions within this subfamily (Figs. 1, 2, and 5). Curiously, in our hands, hDcR3:Fc failed to bind efficiently to its three cognate ligands LIGHT, FasL, and TL1A (data not shown), but the same portion of DcR3 fused to the GPI anchor of TRAIL-R3 did show an interaction (Fig. 1). It has been shown that DcR3 specifically loses its ability to interact with FasL (but not LIGHT) upon proteolytic cleavage at residue Arg-247, which separates the CRDs from the C-terminal extension (35). In contrast to DcR3-Ig, GPI-anchored DcR3 may escape proteolytic processing or maintain a conformation that allows ligand binding. We have not been able to test the hDcR3-mLIGHT interaction.

There were marked differences in the patterns of interaction of the lymphotoxins with their receptors in mouse and human. In the human, both LTα and LTαβ interacted with three receptors each, whereas in the mouse, LTα bound specifically to TNFR1 and LTαβ to LTβR. This raises the question as to whether the interactions of human LTα with HVEM or human LTαβ with TNFR1 are physiologically relevant or whether they represent weak interactions with no real signaling potential *in vivo*.

Fc:ligands allow the detection of weak interactions, because they are predicted to contain more than one trimeric ligand (18) and to display higher avidity for the receptors than regular trimeric soluble ligands. This is illustrated by the unexpected observation that human TNF did bind to mouse TNFR2 in our experimental system (Fig. 1), although it is known that, under physiological conditions, hTNF does not bind and cannot activate mTNFR2 (36, 37). We tested whether this result was because of the increased avidity of Fc:hTNF or to polymorphisms in mTNFR2. Indeed, non-obese diabetic and 129/SvJ mouse strains contain the T102S and I108T polymorphisms (38, 39) (Fig. 3*A*) that could potentially alter the specificity for hTNF as they correspond to amino acids that are present in the ligand binding domain of human TNFR2 (Fig. 3*A*) (16). However, both polymorphic forms of mTNFR2 bound to Fc:hTNF nearly as efficiently as the positive controls hTNFR2, hTNFR1, and mTNFR1 but only weakly bound trimeric human TNF (Fig. 3, *B* and *C*). We conclude that the use of Fc:ligands, which may mimic membrane-bound ligands, contributes to the sensitivity of the screen by allowing detection of weaker interactions. However, additional experiments are required to determine whether a given interaction is biologically relevant.

The BAFF and APRIL Network—The ligands BAFF and APRIL share two receptors, TACI and BCMA. However, only BAFF can bind to BAFF-R. We indeed confirmed these findings with hBAFF and hAPRIL (Fig. 1). Unexpectedly, mAPRIL interacted with mBAFF-R, although the binding was weaker than that observed with mBAFF (Fig. 1). To our knowledge, the binding of mAPRIL to mBAFF-R has not been reported previously in the literature. We noticed that APRIL exists as two splice variants differing by a single amino acid residue, Ala-112, as a result of differential usage of alternative splice acceptor sites that are separated by only three nucleotides (Fig. 4*A*). A similar case has been described for EDA isoforms that differ by two amino acid residues (40). Both APRIL variants were found in mouse expressed sequence tags at approximately equal frequencies and in both "regular" APRIL and in the membrane-bound TWE-PRIL. TWE-PRIL is the result of an intergenic splicing event and contains the entire TNF homology domain of APRIL (41). The APRIL splice variant lacking the Ala-112 (or Asp-112 in human) form cannot be produced in human and was not detected in dog, pig, or cow expressed sequence tags. The available rat expressed sequence tags encode only the short form of APRIL. When expressed as an Fc:ligand, both APRIL splice variants displayed equivalent binding to mTACI and mBCMA, weaker but significant binding to mBAFF-R, and no detectable binding to hBAFF-R (Fig. 4, *B* and *C*). However, only the shorter splice variant bound to mBAFF-R when expressed as FLAG-tagged (trimeric) mAPRIL (Fig. 4, *B* and *C*). This points to a specific interaction between APRIL and BAFF-R in the mouse, which depends in part on the splicing of APRIL. Although this interaction is weak, and despite the fact that the phenotypes of BAFF-deficient, BAFF-R-deficient, and APRIL-deficient mice all indicate that APRIL does not compensate for the loss

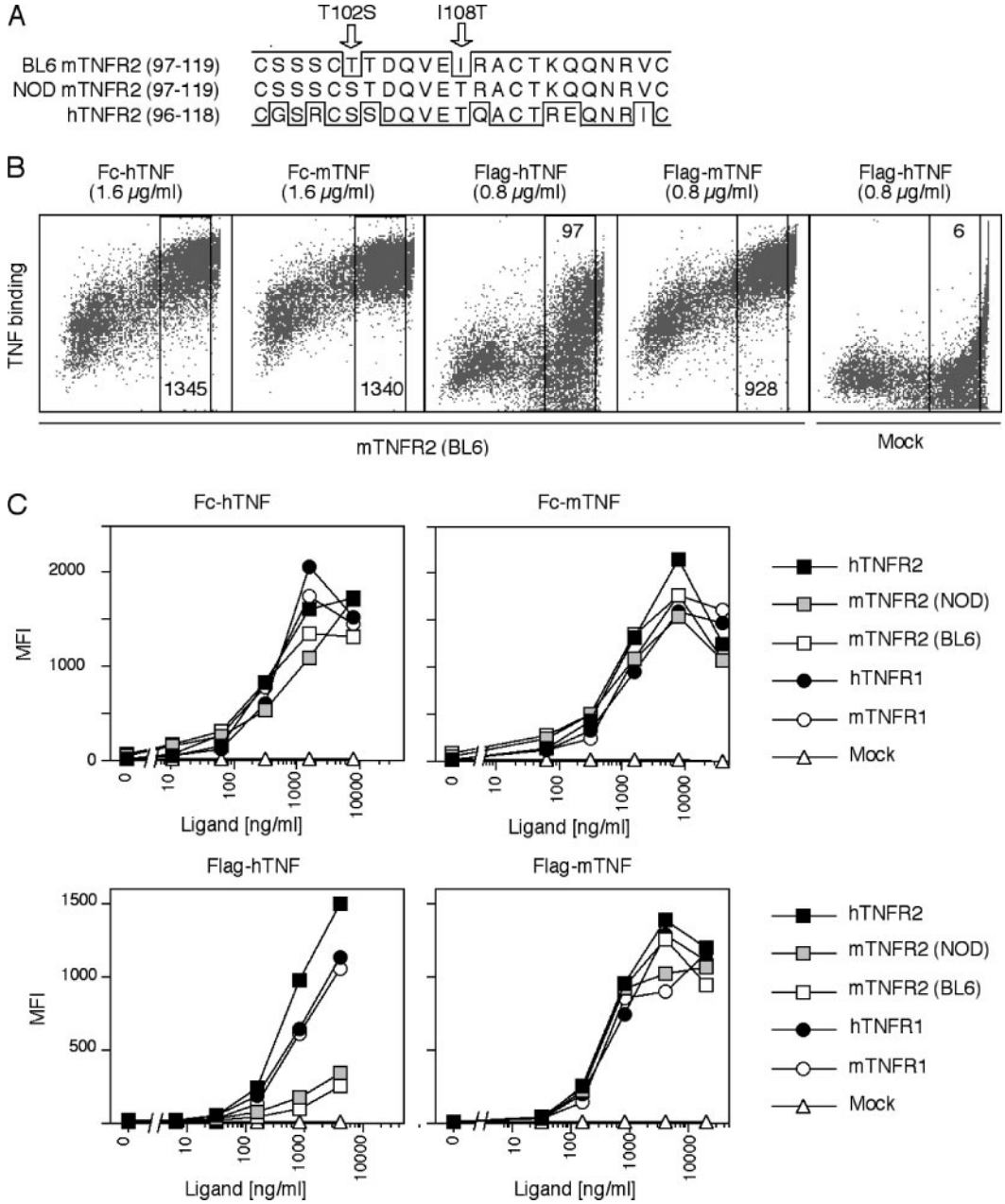

FIGURE 3. **hTNF does not bind polymorphic forms of mTNFR2, unless multimerized as Fc:hTNF.** *A*, alignment of a relevant portion of the second cysteine-rich domain of human and mouse TNFR2-highlighting polymorphisms found in the C57BL/6 (*BL6*) and non-obese diabetic (*NOD*) strains of mice, respectively. *B*, cells were transfected with mTNFR2 or empty plasmid (*Mock*), as described in the legend to Fig. 1, and stained with either Fc:TNF (hexameric) or FLAG-TNF (trimeric). Mean fluorescence intensities (*vertical axis*) of gated, EGFP-positive cells are indicated. Both axes show fluorescence intensity on a logarithmic scale (10^0–10^4). *C*, mean fluorescence intensities (*MFI*) of TNF binding to TNF receptors were measured as described in for *B* and plotted as a function of ligand concentration. Comparable data were obtained in two independent experiments (more for some of the interactions).

of BAFF with regard to BAFF-R activation (9, 42), it remains possible that, in the mouse, APRIL or TWE-PRIL may exert some effects through mBAFF-R. It is also possible that administration of high doses of murine BAFF-R-Ig in the mouse might affect APRIL function in addition to that of BAFF.

We have previously reported that mBCMA-Ig fusion protein expressed at 100 μg/ml in the sera of transgenic mice interacted quite poorly with mBAFF and failed to inhibit the function of BAFF *in vivo* (43). In contrast, the affinity of mBAFF for hBCMA-Ig is high (43) and explains why hBCMA-Ig can block endogenous mBAFF *in vivo* (45, 46). In the present study, we had no difficulty detecting the binding of mBAFF to mBCMA-GPI and full-length mBCMA (Figs. 1 and 4 and data not shown), suggesting that the fusion of mBCMA to an Fc but not to a GPI anchor is detrimental for its binding to mBAFF but curiously not for binding to mAPRIL (43). Fc:mFasL, which binds hDcR3 but not mFas, may represent another example where the fusion to an Ig skews the binding specificity of the ligand (Fig. 1). The lack of interaction between mBAFF and mBCMA-Ig expressed in transgenic mice might therefore be due, at least in part, to an indirect effect of the fusion protein rather than to an intrinsic property of the interaction.

Orphan Receptors and Atypical Interactions—In this screen, all TNFSF members bound to one or more receptors, but five receptors, namely NGFR, DR6, TROY, RELT, and mTNFRH3, remained orphan. As interactions were tested in both human and mouse, it was unlikely

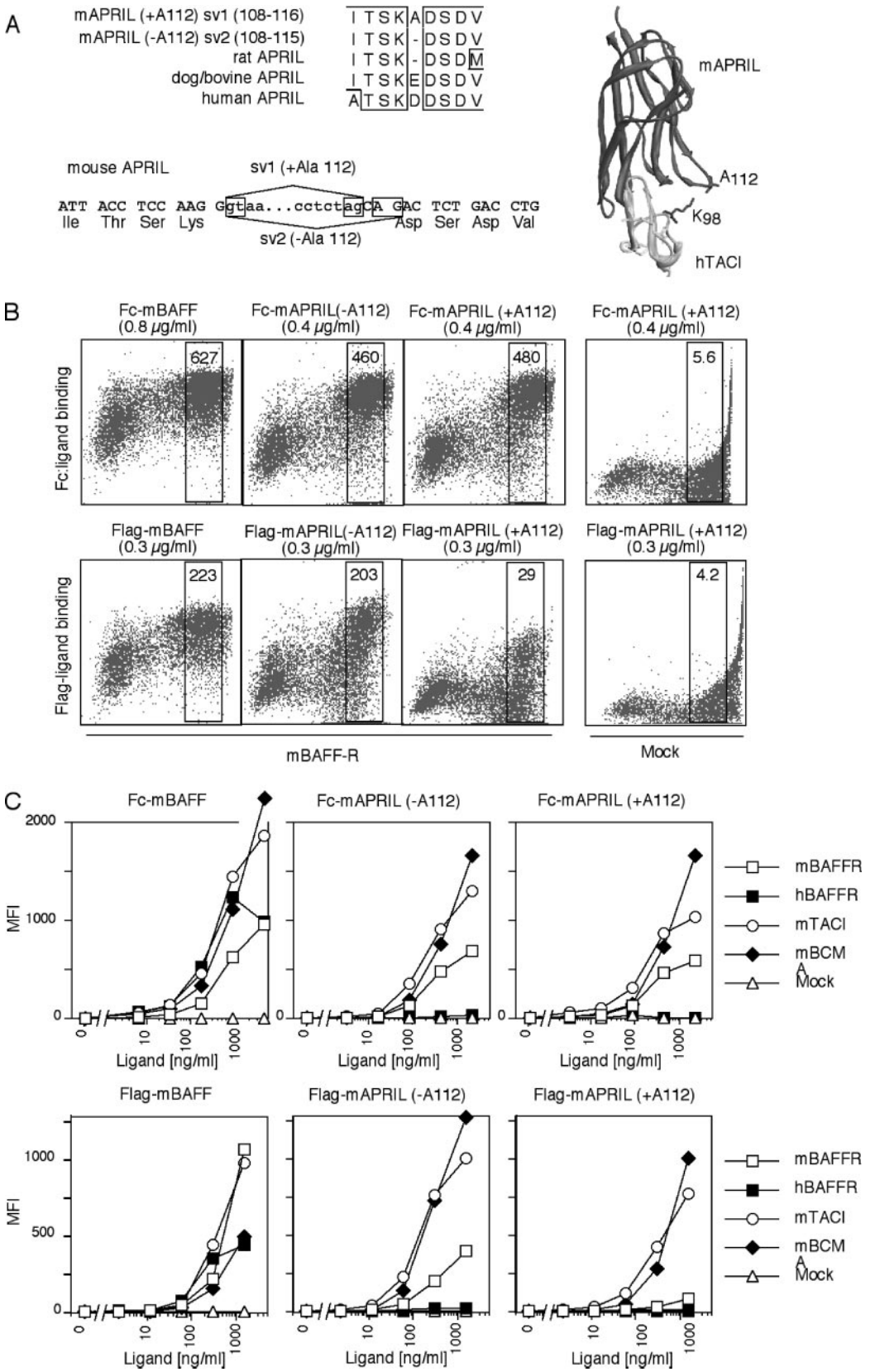

FIGURE 4. **Weak but significant binding of a mAPRIL splice variant to mBAFF-R.** *A*, sequence alignment of APRIL in various species around alanine 112 (*top*), schematic representation of the two splice acceptor sites in the gene of mouse APRIL (*bottom*), and location of Ala-112 in the crystal structure of the mAPRIL- hTACI complex (*right*, based on the Protein Data Bank atomic coordinate file 1XU1) (14). A single monomer is shown. *B*, cells transfected with mBAFF-R or empty plasmid (*Mock*), as described in the legend to Fig. 1, were stained with the indicated concentration of hexameric (Fc fusion, scattergrams) or trimeric (FLAG fusion, *bottom* scattergrams) mBAFF and mAPRIL splice variants. Mean fluorescence intensities (*vertical axis*) of gated, EGFP-positive cells are indicated. Both axes show fluorescence intensity on a logarithmic scale (10^0–10^4). *C*, mean fluorescence intensities (*MFI*) of mBAFF and mAPRIL binding to mBAFF-R, hBAFF-R, mTACI, and mBCMA were measured as described for *B* and plotted as a function of ligand concentration. Comparable data were obtained in two independent experiments. sv1, splice variant 1; sv2, splice variant 2.

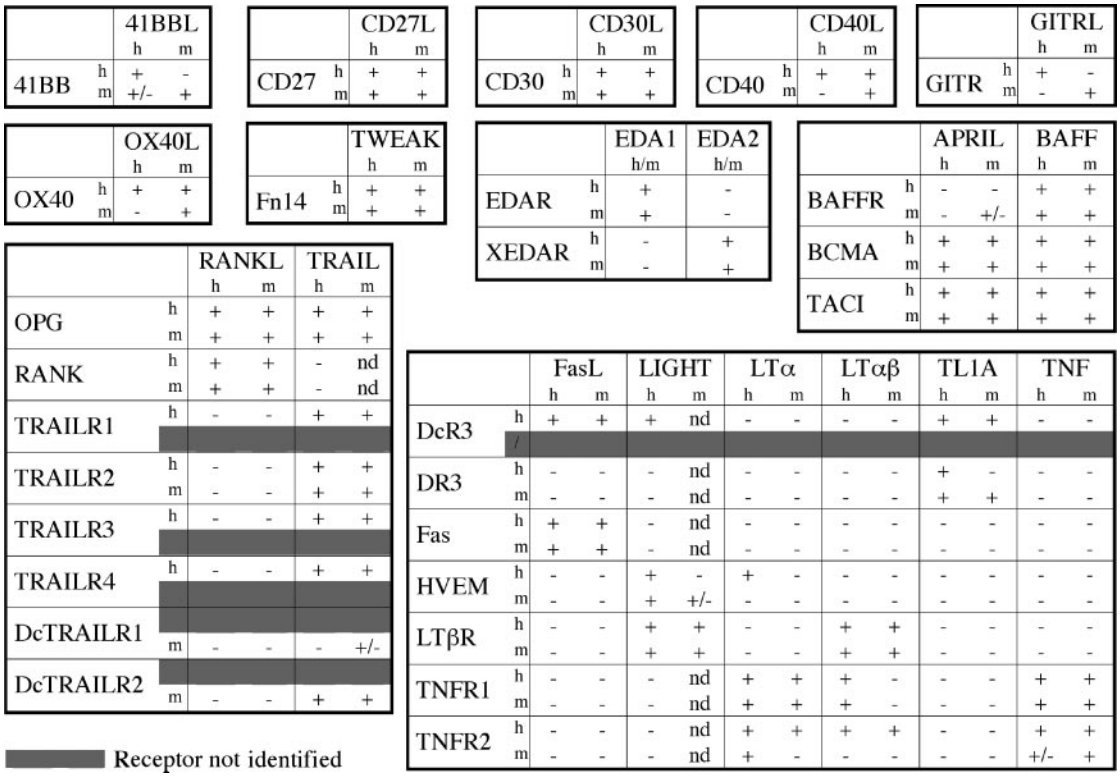

FIGURE 5. **Interactions and species-specificities in the TNF family.** Summary of the interactions detected in this study. Ligands that share at least one receptor are grouped. *h*, human; *m*, mouse; *h/m*, human and mouse have identical sequences; +, interaction; −, no interaction; ±, interaction detected, but weaker than a comparable control. *nd*, not determined. *Shaded areas*, receptor absent or not identified in that species. Detection of a positive interaction *in vitro* does not imply that it is physiologically relevant or that the recombinant ligand is biologically active.

that an interaction with one of the known TNF ligands would have been missed. One possibility is that these receptors may bind heterotrimeric ligands, similar to the LTβR-LTαβ interaction. In addition, we have limited our screen to ligands of the TNF family, although NGFR is known to interact with several dimeric ligands of the neurotrophin family, such as nerve growth factor, brain-derived neurotrophic factor, and other neurotrophins (NT-3, NT-4/5) (47, 48). NGFR and TROY have been reported to interact with membrane-bound partners, such as LINGO-1 and NogoR. In these cases, myelin-derived ligands bind the GPI-anchored NogoR that transmits intracellular signals through the transmembranous protein complexes composed of NGFR, LINGO-1, and NogoR or TROY, LINGO-1, and NogoR (49–51). There are also reports that receptors can function in the absence of ligand (52, 53), and some of the orphan receptors might function uniquely in this manner. Finally, a number of "atypical" interactions have been reported, for example APRIL and TACI with proteoglycans (20, 54), HVEM with the herpes simplex virus glycoprotein D (55), CD40 with Hsp70-peptide complexes (56), TNF with Tanaxapox virus 2L protein, (57), LIGHT with B and T cell attenuator (58, 59), and TNF with specific oligosaccharides (24). None of these interactions would have been uncovered in the present screen.

In summary, our results, collated in Fig. 5, provide a comprehensive survey of interactions and cross-species reactivities within the human and mouse TNF and TNFR families.

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REFERENCES

1. Locksley, R. M., Killeen, N., and Lenardo, M. J. (2001) *Cell* **104,** 487–501
2. Brandt, S. M., Dionne, M. S., Khush, R. S., Pham, L. N., Vigdal, T. J., and Schneider, D. S. (2004) *PLoS Biol.* **2,** e418
3. Kanda, H., Igaki, T., Kanuka, H., Yagi, T., and Miura, M. (2002) *J. Biol. Chem.* **277,** 28372–28375
4. Moreno, E., Yan, M., and Basler, K. (2002) *Curr. Biol.* **12,** 1263–1268
5. Pispa, J., and Thesleff, I. (2003) *Dev. Biol.* **262,** 195–205
6. Tamada, K., and Chen, L. (2006) *Cancer Immunol. Immunother.* **55,** 355–362
7. Feldmann, M., Brennan, F. M., Foxwell, B. M., Taylor, P. C., Williams, R. O., and Maini, R. N. (2005) *J. Autoimmun.* **25,** (suppl.) 26–28
8. Kelley, S. K., and Ashkenazi, A. (2004) *Curr. Opin. Pharmacol.* **4,** 333–339
9. Kalled, S. L. (2005) *Immunol. Rev.* **204,** 43–54
10. Tanaka, S., Nakamura, K., Takahasi, N., and Suda, T. (2005) *Immunol. Rev.* **208,** 30–49
11. Bodmer, J. L., Schneider, P., and Tschopp, J. (2002) *Trends Biochem. Sci.* **27,** 19–26
12. Browning, J. L., Ngam-ek, A., Lawton, P., DeMarinis, J., Tizard, R., Chow, E. P., Hession, C., O'Brine-Greco, B., Foley, S. F., and Ware, C. F. (1993) *Cell* **72,** 847–856
13. Liu, Y., Hong, X., Kappler, J., Jiang, L., Zhang, R., Xu, L., Pan, C. H., Martin, W. E., Murphy, R. C., Shu, H. B., Dai, S., and Zhang, G. (2003) *Nature* **423,** 49–56
14. Hymowitz, S. G., Patel, D. R., Wallweber, H. J., Runyon, S., Yan, M., Yin, J., Shriver, S. K., Gordon, N. C., Pan, B., Skelton, N. J., Kelley, R. F., and Starovasnik, M. A. (2005) *J. Biol. Chem.* **280,** 7218–7227
15. Hymowitz, S. G., Christinger, H. W., Fuh, G., Ultsch, M., O'Connell, M., Kelley, R. F., Ashkenazi, A., and de Vos, A. M. (1999) *Mol. Cell* **4,** 563–571
16. Banner, D. W., D'Arcy, A., Janes, W., Gentz, R., Schoenfeld, H. J., Broger, C., Loetscher, H., and Lesslauer, W. (1993) *Cell* **73,** 431–445
17. Theoleyre, S., Wittrant, Y., Tat, S. K., Fortun, Y., Redini, F., and Heymann, D. (2004) *Cytokine Growth Factor Rev.* **15,** 457–475
18. Holler, N., Tardivel, A., Kovacsovics-Bankowski, M., Hertig, S., Gaide, O., Martinon, F., Tinel, A., Deperthes, D., Calderara, S., Schulthess, T., Engel, J., Schneider, P., and Tschopp, J. (2003) *Mol. Cell Biol.* **23,** 1428–1440
19. Schneider, P., Bodmer, J. L., Holler, N., Mattmann, C., Scuderi, P., Terskikh, A., Peitsch, M. C., and Tschopp, J. (1997) *J. Biol. Chem.* **272,** 18827–18833
20. Ingold, K., Zumsteg, A., Tardivel, A., Huard, B., Steiner, Q. G., Cachero, T. G., Qiang, F., Gorelik, L., Kalled, S. L., Acha-Orbea, H., Rennert, P. D., Tschopp, J., and Schneider, P. (2005) *J. Exp. Med.* **201,** 1375–1383

21. Schneider, P. (2000) *Methods Enzymol.* **322,** 322–345
22. Schneider, P., Bodmer, J. L., Thome, M., Hofmann, K., Holler, N., and Tschopp, J. (1997) *FEBS Lett.* **416,** 329–334
23. Legler, D. F., Doucey, M. A., Schneider, P., Chapatte, L., Bender, F. C., and Bron, C. (2005) *FASEB J.* **19,** 73–75
24. Sherblom, A. P., Decker, J. M., and Muchmore, A. V. (1988) *J. Biol. Chem.* **263,** 5418–5424
25. Bodmer, J. L., Meier, P., Tschopp, J., and Schneider, P. (2000) *J. Biol. Chem.* **275,** 20632–20637
26. Hymowitz, S. G., O'Connell, M. P., Ultsch, M. H., Hurst, A., Totpal, K., Ashkenazi, A., de Vos, A. M., and Kelley, R. F. (2000) *Biochemistry* **39,** 633–640
27. Pitti, R. M., Marsters, S. A., Lawrence, D. A., Roy, M., Kischkel, F. C., Dowd, P., Huang, A., Donahue, C. J., Sherwood, S. W., Baldwin, D. T., Godowski, P. J., Wood, W. I., Gurney, A. L., Hillan, K. J., Cohen, R. L., Goddard, A. D., Botstein, D., and Ashkenazi, A. (1998) *Nature* **396,** 699–703
28. Marsters, S. A., Sheridan, J. P., Pitti, R. M., Brush, J., Goddard, A., and Ashkenazi, A. (1998) *Curr. Biol.* **8,** 525–528
29. Kaptein, A., Jansen, M., Dilaver, G., Kitson, J., Dash, L., Wang, E., Owen, M. J., Bodmer, J. L., Tschopp, J., and Farrow, S. N. (2000) *FEBS Lett.* **485,** 135–141
30. Schneider, P., Schwenzer, R., Haas, E., Muhlenbeck, F., Schubert, G., Scheurich, P., Tschopp, J., and Wajant, H. (1999) *Eur. J. Immunol.* **29,** 1785–1792
31. Nakayama, M., Ishidoh, K., Kayagaki, N., Kojima, Y., Yamaguchi, N., Nakano, H., Kominami, E., Okumura, K., and Yagita, H. (2002) *J. Immunol.* **168,** 734–743
32. Wiley, S. R., Cassiano, L., Lofton, T., Davis-Smith, T., Winkles, J. A., Lindner, V., Liu, H., Daniel, T. O., Smith, C. A., and Fanslow, W. C. (2001) *Immunity* **15,** 837–846
33. Migone, T. S., Zhang, J., Luo, X., Zhuang, L., Chen, C., Hu, B., Hong, J. S., Perry, J. W., Chen, S. F., Zhou, J. X., Cho, Y. H., Ullrich, S., Kanakaraj, P., Carrell, J., Boyd, E., Olsen, H. S., Hu, G., Pukac, L., Liu, D., Ni, J., Kim, S., Gentz, R., Feng, P., Moore, P. A., Ruben, S. M., and Wei, P. (2002) *Immunity* **16,** 479–492
34. Schneider, P., Olson, D., Tardivel, A., Browning, B., Lugovskoy, A., Gong, D., Dobles, M., Hertig, S., Hofmann, K., Van Vlijmen, H., Hsu, Y. M., Burkly, L. C., Tschopp, J., and Zheng, T. S. (2003) *J. Biol. Chem.* **278,** 5444–5454
35. Wroblewski, V. J., McCloud, C., Davis, K., Manetta, J., Micanovic, R., and Witcher, D. R. (2003) *Drug Metab. Dispos.* **31,** 502–507
36. Ranges, G. E., Bombara, M. P., Aiyer, R. A., Rice, G. G., and Palladino, M. A., Jr. (1989) *J. Immunol.* **142,** 1203–1208
37. Ameloot, P., Fiers, W., De Bleser, P., Ware, C. F., Vandenabeele, P., and Brouckaert, P. (2001) *J. Biol. Chem.* **276,** 37426–37430
38. Hurle, B., Segade, F., Rodriguez, R., Ramos, S., and Lazo, P. S. (1998) *Genomics* **52,** 79–89
39. Powell, E. E., Wicker, L. S., Peterson, L. B., and Todd, J. A. (1994) *Mamm. Genome* **5,** 726–727
40. Yan, M., Wang, L. C., Hymowitz, S. G., Schilbach, S., Lee, J., Goddard, A., de Vos, A. M., Gao, W. Q., and Dixit, V. M. (2000) *Science* **290,** 523–527
41. Pradet-Balade, B., Medema, J. P., Lopez-Fraga, M., Lozano, J. C., Kolfschoten, G. M., Picard, A., Martinez, A. C., Garcia-Sanz, J. A., and Hahne, M. (2002) *EMBO J.* **21,** 5711–5720
42. Schneider, P. (2005) *Curr. Opin. Immunol.* **17,** 282–289
43. Schneider, P., Takatsuka, H., Wilson, A., Mackay, F., Tardivel, A., Lens, S., Cachero, T. G., Finke, D., Beermann, F., and Tschopp, J. (2001) *J. Exp. Med.* **194,** 1691–1697
44. Deleted in proof
45. Thompson, J. S., Schneider, P., Kalled, S. L., Wang, L., Lefevre, E. A., Cachero, T. G., MacKay, F., Bixler, S. A., Zafari, M., Liu, Z. Y., Woodcock, S. A., Qian, F., Batten, M., Madry, C., Richard, Y., Benjamin, C. D., Browning, J. L., Tsapis, A., Tschopp, J., and Ambrose, C. (2000) *J. Exp. Med.* **192,** 129–135
46. Pelletier, M., Thompson, J. S., Qian, F., Bixler, S. A., Gong, D., Cachero, T., Gilbride, K., Day, E., Zafari, M., Benjamin, C., Gorelik, L., Whitty, A., Kalled, S. L., Ambrose, C., and Hsu, Y. M. (2003) *J. Biol. Chem.* **278,** 33127–33133
47. He, X. L., and Garcia, K. C. (2004) *Science* **304,** 870–875
48. Nykjaer, A., Willnow, T. E., and Petersen, C. M. (2005) *Curr. Opin. Neurobiol.* **15,** 49–57
49. Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., Levesque, M., Allaire, N., Perrin, S., Sands, B., Crowell, T., Cate, R. L., McCoy, J. M., and Pepinsky, R. B. (2004) *Nat. Neurosci.* **7,** 221–228
50. Shao, Z., Browning, J. L., Lee, X., Scott, M. L., Shulga-Morskaya, S., Allaire, N., Thill, G., Levesque, M., Sah, D., McCoy, J. M., Murray, B., Jung, V., Pepinsky, R. B., and Mi, S. (2005) *Neuron* **45,** 353–359
51. Park, J. B., Yiu, G., Kaneko, S., Wang, J., Chang, J., He, X. L., Garcia, K. C., and He, Z. (2005) *Neuron* **45,** 345–351
52. Micheau, O., Solary, E., Hammann, A., and Dimanche-Boitrel, M. T. (1999) *J. Biol. Chem.* **274,** 7987–7992
53. Shrivastava, P., Pantano, C., Watkin, R., McElhinney, B., Guala, A., Poynter, M. L., Persinger, R. L., Budd, R., and Janssen-Heininger, Y. (2004) *Mol. Cell. Biol.* **24,** 6763–6772
54. Bischof, D., Elsawa, S. F., Mantchev, G., Yoon, J., Michels, G. E., Nilson, A., Sutor, S., Platt, J. L., Ansell, S. M., von Bulow, G., and Bram, R. J. (2006) *Blood* **107,** 3235–3242
55. Carfi, A., Willis, S. H., Whitbeck, J. C., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Wiley, D. C. (2001) *Mol. Cell* **8,** 169–179
56. Becker, T., Hartl, F. U., and Wieland, F. (2002) *J. Cell Biol.* **158,** 1277–1285
57. Brunetti, C. R., Paulose-Murphy, M., Singh, R., Qin, J., Barrett, J. W., Tardivel, A., Schneider, P., Essani, K., and McFadden, G. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100,** 4831–4836
58. Sedy, J. R., Gavrieli, M., Potter, K. G., Hurchla, M. A., Lindsley, R. C., Hildner, K., Scheu, S., Pfeffer, K., Ware, C. F., Murphy, T. L., and Murphy, K. M. (2005) *Nat. Immunol.* **6,** 90–98
59. Compaan, D. M., Gonzalez, L. C., Tom, I., Loyet, K. M., Eaton, D., and Hymowitz, S. G. (2005) *J. Biol. Chem.* **280,** 39553–39561