Studies on the interaction between TWEAK and the death receptor WSL-1/TRAMP (DR3)

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Abstract WSL-1/TRAMP (DR3) is a member of the tumour necrosis factor (TNF) receptor superfamily which exhibits effects on NF-κB activation and apoptosis. TWEAK, a novel TNF-related molecule, has been proposed as the ligand for this receptor. Utilising both human and murine TWEAK ligand, it is shown that TWEAK and WSL-1/TRAMP do not interact in an in vitro binding assay and that TWEAK binds strongly to cells that do not express WSL-1/TRAMP on the cell surface. Biological activity of TWEAK is also observed in these cells. Finally, cells isolated from WSL-1/TRAMP knockout mice are shown to retain their ability to interact with TWEAK. These results suggest that WSL-1/TRAMP is not the major receptor for TWEAK

Key words: TWEAK; WSL-1; TRAMP; Death receptor; Apoptosis

1. Introduction

The tumour necrosis factor (TNF) ligand and receptor superfamilies play a variety of key biological roles, including regulation of apoptosis, cellular differentiation and inflammation [1–6]. The ligands are mostly expressed as type 2 membrane proteins, however many of these can also be produced as soluble cytokines by proteolysis. The receptors are characterised by the presence of a variable number of cysteine-rich motifs in the extracellular domain. The cytoplasmic regions of these receptors are much less conserved, except for a subgroup characterised by the presence of an 80 amino acid death domain. This motif is responsible for recruiting the intracellular signalling machinery leading in the case of apoptosis to activation of a caspase cascade [7].

Recently, database searching utilising conserved sequence motifs and the application of techniques for studying protein–protein interactions has led to the discovery of many new members of this family [1,3]. Several years ago, we and others identified a novel death domain containing receptor WSL-1/TRAMP (DR3, APO-3, LARD) [8–12], which has preferential expression in tissues of the immune system. This receptor is a potent inducer of apoptosis and can also activate the transcription factor NF-κB. The extracellular domain of this receptor failed to bind with known members of the ligand family, suggesting that WSL-1/TRAMP had a novel cognate ligand. It has been proposed since that the novel TNF family member TWEAK is the ligand for WSL-1/TRAMP [13]. TWEAK has been previously identified as a weak inducer of apoptosis, and can also mediate chemokine production and endothelial cell proliferation [14–16]. Therefore we have performed an extensive series of experiments to explore this observation.

Firstly we performed a series of binding studies to determine the affinity of WSL-1/TRAMP for a large panel of TNF-related ligands, including TWEAK. Secondly we studied the effect of TWEAK on cells overexpressing the WSL-1/TRAMP receptor. We also studied the effect of TWEAK on several untransfected cell lines including cells which do not express WSL-1/TRAMP. Finally we cloned the murine TWEAK ligand and studied its binding to peripheral blood lymphocytes from normal and WSL-1/TRAMP knockout mice.

2. Materials and methods

2.1. Constructs

The complete cDNA coding sequence for WSL-1/TRAMP was cloned into pCDNA3 (Invitrogen). For the expression of soluble mTWEAK and hTWEAK the sequence encoding the extracellular region was cloned into pFLAG-CMV1 vector (Kodak-ABI) 3′ to a preprotrypsin leader sequence and FLAG epitope. Expression of the receptor:Fc fusion protein has been previously described [11]. Activation of NF-κB was studied using the promoter reporter construct p(NF-κB)4-tk-sPAP, containing four κB response elements (GGACTTTCC) upstream of the minimal thymidine kinase promoter and the cDNA sequence for secreted placental alkaline phosphatase. The extracellular domains of WSL-1/TRAMP (aa 1–181) and TRAIL-R2 (aa 1–212) were fused to the C-terminal portion of TRAIL-R3 (aa 157–259) containing the TAPE repeats and GPI addition signal sequence via a 3 aa. linker (VDK, *Sal*I site). Stable clones were established in 293 cells and positive clones were selected by FACS staining.

2.2. Cell culture

HEK293, HEK293T, A375, HT29 cell lines were obtained from the ATCC. The cells were cultured in Dulbecco's minimal essential medium (DMEM) (Gibco BRL Ltd), supplemented with 10% foetal calf serum (FCS), 2 mM glutamine, penicillin (100 IU/ml), and streptomycin (100 μg/ml).

Primary murine cells used in the different studies were isolated from blood (citrate plasma). In the FACS analysis cells were gated on lymphocytes.

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2.3. Transfections

HEK293 and HEK293T cells were transfected using Lipofectamine (Gibco BRL) according to the manufacturer's protocol. For the HEK293T cells transfected with WSL-1/TRAMP a total of 1.4 μg of DNA per well of a 6 well plate was used (6×10^5 cells). The amount of pCDNA3/WSL-1 varied between 0 and 0.5 μg per well, 0.4 μg of reporter construct p(NF-κB)4-tk-sPAP was used per well. The promoter reporter construct RSV–lactamase was cotransfected, to control for possible differences in transfection efficiency and to allow the determination of the effect of overexpression of WSL-1/TRAMP on induction of apoptosis. The total amount of DNA in the transfections was kept constant by the addition of the empty expression vector.

For HEK293 cells 10 μg pCMV1-FLAG-TWEAK plasmid per 75 cm^2 flask was used (70–80% confluency).

SPAP and lactamase activity were quantified by standard colourimetric assay.

2.4. Purification of TWEAK protein

24 h after transfection of HEK293 cells with either murine or human TWEAK in pFLAG-CMV1, the medium was changed to the serum free medium OPTIMEM. Following an additional 24 h incu-

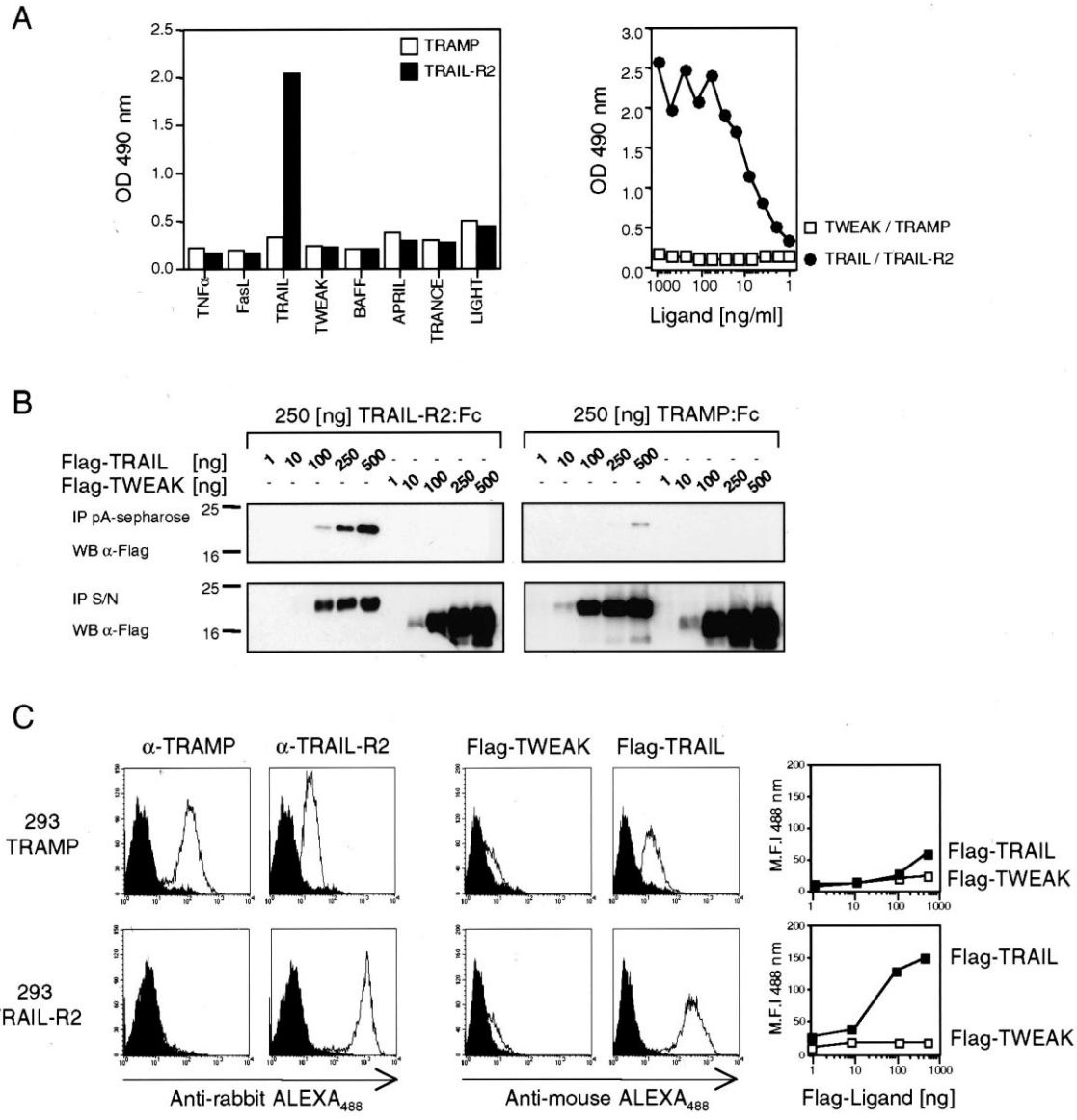

Fig. 1. Recombinant TWEAK/Apo-3L does not bind TRAMP. A: Interaction of TNF ligands with TRAMP and TRAIL-R2. Wells of microtiterplates were coated with TRAMP:Fc (white bars) and TRAIL-R2:Fc (black bars). Binding of the Flag-tagged-ligands (1 μg/ml) was determined by the addition of anti-Flag M2 antibody followed by anti-mouse-HRP conjugate. The dose dependence of the TRAIL:TRAIL-R2 interaction was analysed by titration of TRAIL in the right panel. B: Lack of immunoprecipitation (IP) between TWEAK and TRAMP. Recombinant TRAIL-R2:Fc or TRAMP:Fc (250 ng) were mixed with the indicated amounts of recombinant Flag-TRAIL and Flag-TWEAK respectively and subsequently immunoprecipitated with PA-Sepharose. The IPs (upper panels) and supernatants (lower panels) were analysed by anti-Flag Western blot. No binding was observed using unpurified TRAMP:Fc. C: TWEAK does not interact with TRAMP expressed at the surface of cells. 293 cells stably transfected with either TRAMP:GPI (upper panels) or TRAIL-R2:GPI (lower panels) were stained with rabbit anti-TRAMP polyclonal antibody (AL158), rabbit anti-TRAIL-R2 polyclonal antibody (AL142), Flag-TWEAK (100 ng) and Flag-TRAIL (100 ng). Polyclonal antibodies were revealed using anti-rabbit Fab′2 coupled to ALEXA488 and Flag ligands were revealed using anti-Flag M2 followed by anti-mouse Fab′2 coupled to ALEXA488. The black histograms represent control staining either with non-specific rabbit polyclonal IgG (for TRAMP or TRAIL-R2) or absence of flagged ligand (Flag-TWEAK and Flag-TRAIL). The right panels show the dose–response curves of the interactions.

bation, the medium was harvested. The FLAG-tagged, secreted TWEAK present in culture medium was purified using a M2-agarose column (Sigma) according to the manufacturer's protocol.

2.5. FACS analysis

2.5.1. WSL-1/TRAMP cell surface expression. Cells were harvested in cell dissociation buffer (Sigma). For the incubations with antibodies, the buffer was changed to FACS buffer (PBS containing 2.5% FCS and 1% azide). 1×10^6 cells were incubated for 15 min at RT in the presence or absence of 3 μg rabbit polyclonal antibody against WSL-1/TRAMP (Serotec). Cells were rinsed with FACS buffer and incubated for 15 min at RT with 0.5 μg anti-rabbit Alexa 488. After a final wash cells were resuspended in 0.5 ml FACS buffer and analysed by flow cytometry (Beckman-Coulter XL bench top flow cytometer).

2.5.2. TWEAK binding. For the binding of TWEAK to HT29 and A375 cells the procedure is essentially as described above, with the addition of an incubation in the presence or absence of the ligand. In the first incubation cells were incubated for 10 min in the presence or absence of 1–1000 ng per stain of FLAG-mTWEAK, FLAG-hTWEAK or FLAG-hTRAIL. This is followed by a 10 min incubation, with 0.5 μg anti-FLAG (M2, Sigma) and finally a 10 min incubation with 0.5 μg anti-mouse Alexa 488.

For the TWEAK binding to primary murine cells, after the incubation in the presence or absence of FLAG-mTWEAK (3 μg per incubation), cells were incubated with normal mouse serum to block murine Fc-receptors and then stained with 0.5 μg anti-FLAG conjugated to biotin (M2-biotin, Sigma). In the last incubation 0.5 μg Streptavidin Alexa 488 was used.

2.5.3. Double staining of murine cells. To identify cell types binding to TWEAK, ligand binding studies were performed as described above, with the addition in the last incubation of phycoerythrin conjugates of antibodies (0.4 μg for each conjugate) for cell specific proteins (anti-CD3, T-cells; anti-CD45-R, B-cells; anti-CD14, monocytes/macrophages). Hamster IgG2A-PE was used as an isotype control and normal mouse serum was added to block murine Fc-receptors.

2.6. In vitro binding assays

2.6.1. Ligand binding ELISA. Wells of 96 well ELISA plates (Nunc Maxisorp, 439454) were coated with various receptor:Fc recombinant fusion constructs (1 μg/ml in PBS, 100 μl, 2–16 h, 37°C). After saturation in block buffer (PBS containing 5% FCS, 1 h, 37°C) and three washes (PBS containing 0.05% Tween 20) using an ELISA washer, Flag-tagged ligands were added (1 μg/ml in PBS containing 0.5% FCS, 100 μl, 1 h, 37°C) and serially diluted in PBS as indicated. After three washes (PBS, 0.05% Tween-20) bound ligands were revealed with anti-Flag M2 antibody (1 μg/ml in block buffer, 100 μl, 30 min, 37°C), rabbit anti-mouse IgG coupled to peroxidase (1/1000 dilution in block buffer, 100 μl, 30 min, 37°C) and *o*-phenylenediamine hydrochloride (0.3 mg/ml in 50 mM citric acid, 100 mM Na_2HPO_4, 0.01% H_2O_2). The plate was washed four times between each step of the detection. Absorbance was measured at 490 nm with an ELISA reader.

2.6.2. Immunoprecipitation. 250 ng of purified recombinant TRAMP:Fc or TRAIL-R2:Fc were mixed with 1, 10, 100, 250 and 500 ng Flag-TRAIL or Flag-TWEAK in 200 μl of PBS containing 10 μg BSA. The mixture was precleared for 1 h on 10 μl Sepharose-6B beads and the supernatant was immunoprecipitated overnight with 10 μl PA-Sepharose. The beads were washed three times with 500 μl PBS and proteins were eluted by boiling 5 min in SDS sample buffer containing 100 mM DTT and analysed by SDS–PAGE and Western blot.

2.7. IL-8 ELISA

IL-8 protein was measured using a standard ELISA procedure.

3. Results

3.1. Interaction of WSL-1/TRAMP with TNF family ligands

It has been shown previously, using an ELISA-based in vitro assay, that TRAMP does not interact with FasL, TRAIL and $LT\alpha_1\beta_2$. This initial experiment was repeated, using a larger panel of TNF-related ligands, including the recently characterised BAFF, APRIL, TRANCE/RANKL, LIGHT and TWEAK. No significant binding of any of these ligands to WSL-1/TRAMP was observed, while TRAIL binding to TRAIL-R2 was easily detected under the same set of conditions (Fig. 1A). To exclude the possibility that the TWEAK-WSL-1/TRAMP interaction is of low affinity, and hence only detectable at very high ligand concentrations, a titration experiment was performed using the same setup. In our hands, no TWEAK binding to TRAMP was detected at ligand concentrations as high as 1 μg/ml, whereas 20-fold lower TRAIL concentrations were already sufficient to yield a saturating signal on TRAIL-R2 (Fig. 1A). Purification of

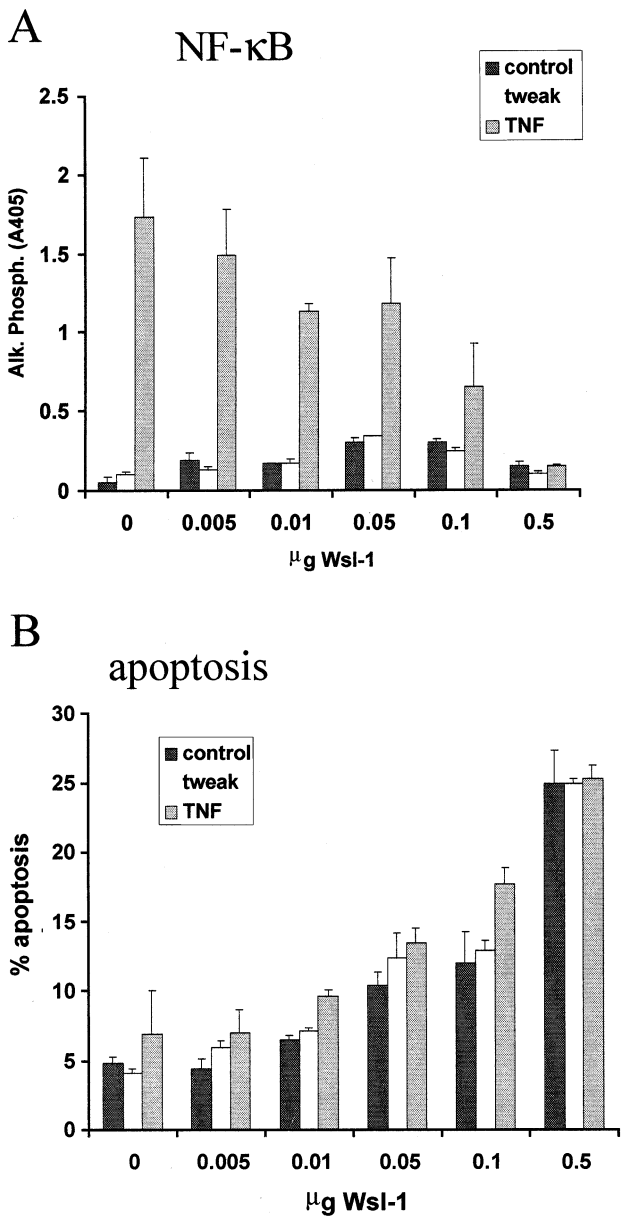

Fig. 2. TWEAK has no effect on apoptosis or NF-κB activation induced by WSL-1/TRAMP. HEK293T cells were transfected with the indicated concentrations of a pCDNA3 WSL-1 expression vector in the presence or absence of recombinant TNFα or TWEAK (10 ng/ml). After 16 h incubation alkaline phosphatase activity was measured using a colourimetric assay (A), apoptosis (B) was estimated by propidium iodide staining and FACS analysis.

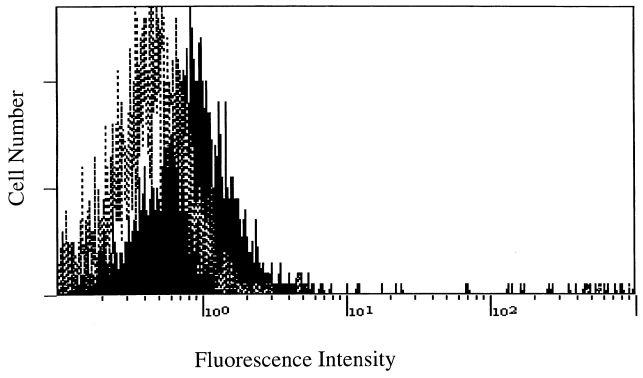

Fig. 3. Transfected WSL-1/TRAMP is expressed on the cell surface of HEK293T cells. 20 h following transfection of 6×10^5 293T cells with 0.1 μg pCDNA3 WSL-1 expression construct the cells were harvested and stained with rabbit polyclonal anti-WSL-1/DR3 and the stained cells detected by flow cytometry (black histogram). Control untransfected cells were treated similarly (dotted histogram).

TRAMP:Fc or its adsorption to plastic might result in its denaturation and loss of binding capacity, and thus a parallel binding experiment was performed using recombinant TRAMP:Fc (purified or as crude cell supernatant) and Flag-tagged TWEAK in solution. Consistent with the ELISA assay, no TWEAK was co-immunoprecipitated using TRAMP:Fc whereas TRAIL efficiently bound to TRAIL-R2:Fc (Fig. 1B). Finally, ligand binding was performed on HEK 293 clones, stably transfected with expression vectors encoding glycosylphosphatidylinositol (GPI)-anchored receptor fusion proteins. Surface expression of TRAMP:GPI and TRAIL-R2:GPI was analysed by flow cytometry using polyclonal anti-TRAIL-R2 and anti-TRAMP antibodies (Fig. 1C). It is noteworthy that the TRAMP:GPI expressing clone displayed significant staining for TRAIL-R2, probably reflecting the endogenous level of TRAIL-R2. Both clones were not stained with Flag-TWEAK, whereas Flag-TRAIL staining correlated completely with TRAIL-R2 expression levels on both clones as detected by anti-TRAIL-R2 antibodies.

3.2. Interaction of TWEAK with WSL-1/TRAMP expressing cells

Both TWEAK and WSL-1/TRAMP are capable of activating NF-κB and inducing apoptosis [8,11,16], and it has been previously reported that transfection of WSL-1 (Apo 3) into HEK293 cells sensitises these cells to apoptosis mediated by TWEAK [5]. HEK293T cells were transfected with varying

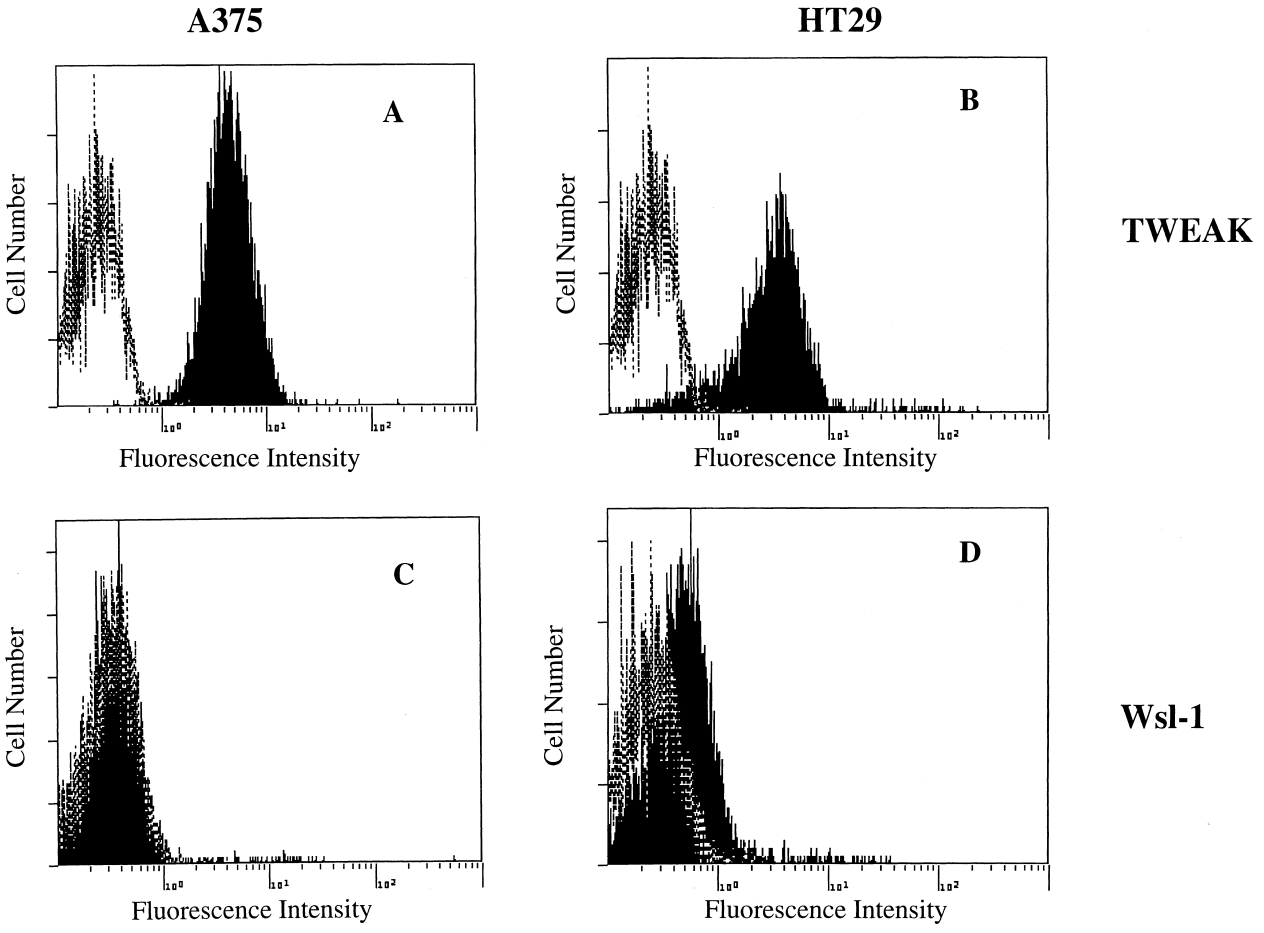

Fig. 4. Binding of TWEAK and WSL-1 to A375 and HT29 cells. Cells were incubated for 10 min in the presence (black histogram) or absence (dotted histogram) of 1 μg FLAG-tagged murine or human TWEAK (A and B). This was followed by a 10 min incubation with 0.5 μg anti-FLAG antibody and finally a 10 min incubation with 0.5 μg anti-mouse Alexa488. For WSl-1 binding (C and D) the cells were incubated for 10 min with 3 μg rabbit polyclonal anti -WSL-1/TRAMP antibody (black histogram). This was followed by a 10 min incubation with 0.5 μg anti-rabbit Alexa488. In all cases the cells were then washed in FACS buffer and analysed by flow cytometry. Control were treated in the same way, except that anti-WSL-1 was omitted (dotted histogram).

concentrations of a WSL-1/TRAMP expression vector and NF-κB activation and apoptosis determined following treatment with or without recombinant TWEAK or TNFα. As shown in Fig. 2, the transfection with WSL-1/TRAMP induced the activation of NF-κB and at higher concentrations of the expression vector increasing levels of apoptosis were induced. Treatment with TWEAK had no effect on the activation of NF-κB or apoptosis induced by WSL-1/TRAMP. WSL-1 and TNF-R1 are reported to use the same intracellular signalling molecules. Incubation with TNFα resulted in a strong activation of NF-κB in the presence or absence of WSL-1, indicating that the signalling pathways downstream of WSL-1 and TNFR1 are functional in these cells. NF-κB activation by TNFα was decreased at higher concentration of the WSL-1/TRAMP expression vector because of WSL-1-induced apoptosis. Expression of WSL-1/TRAMP on the cell surface of the transfected 293T cells was confirmed by FACS analysis using a polyclonal antibody against WSL-1 (Fig. 3) and therefore does not explain why we did not observe the previously reported synergistic effect of TWEAK and WSL-1 on apoptosis.

3.3. Interaction of TWEAK with A375 and HT29 cells

In order to establish that the recombinant TWEAK was biologically active, we treated HT29 and A375 cells with TWEAK and observed the expected stimulation of IL-8 (Table 1). Additionally we observed rapid induction of apoptosis in HT29 cells treated with TWEAK in the presence of interferon γ (data not shown). Binding of murine TWEAK to both A375 and HT29 cells was observed (Fig. 4a,b), identical results were found with recombinant human TWEAK (data not shown). In particular, strong binding to A375 cells was observed. To establish the presence of the WSL-1/TRAMP receptor on the surface of these cells we used an antibody against the extracellular domain of WSL-1, and observed that the receptor is only expressed on the surface of HT29 cells (Fig. 4c,d).

These observations, namely the strong binding and biological activity of TWEAK in A375 cells, which do not express the WSL-1/TRAMP receptor, suggest that there is either another receptor for TWEAK on A375 cells, or that WSL-1/TRAMP is not the true TWEAK receptor. Therefore we chose to extend these studies into a system where we could study the effect of TWEAK in the complete absence of WSL-1/TRAMP.

3.4. Binding of murine TWEAK to peripheral blood lymphocytes from wild-type and WSL-1/TRAMP knockout mice

Knockout mice in which the WSL-1/TRAMP receptor is deleted have been generated (submitted for publication). Peripheral blood lymphocytes from both wild-type and homozygous mice were isolated and TWEAK binding studies performed. No significant differences were observed in the ability of TWEAK to bind to either WSL-1 +/+, +/− or −/− PBL's (Fig. 5). Positive binding percentages were 34, 21 and 29% respectively. Further analysis was performed to establish which cells bound to TWEAK in these experiments. Utilising

Table 1

	IL-8 concentration medium (pg/ml)			
	HT29 cells		A375 cells	
	−TWEAK	+TWEAK	−TWEAK	+TWEAK
0–24 h	718	999	230	584
24–48 h	778	970	278	614

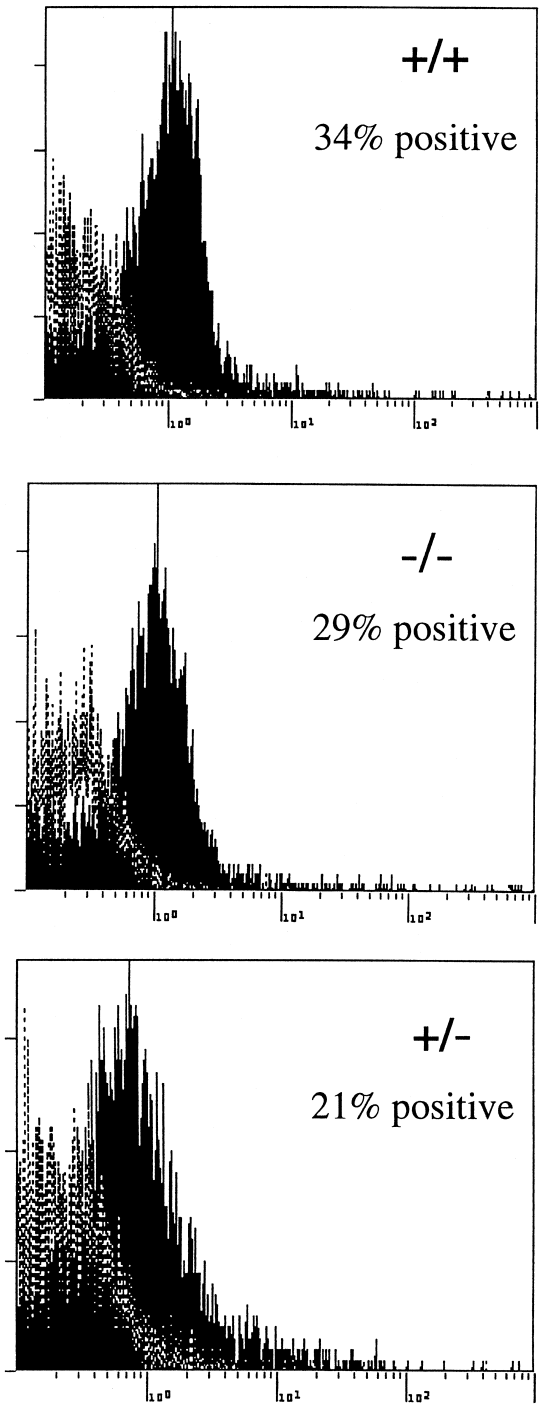

Fig. 5. TWEAK binds to both murine wild-type and WSL-1/TRAMP knockout cells. Peripheral blood lymphocytes from wild-type and wsl-1 knockout mice were incubated for 10 min in the presence or absence of FLAG-tagged murine TWEAK (3 μg). Cells were then incubated with 0.5 μg anti-FLAG conjugated to biotin, following the blocking of murine Fc receptors with normal mouse serum. Finally the cells were incubated with 0.5 μg Streptavidin Alexa488, washed in FACS buffer and analysed by flow cytometry.

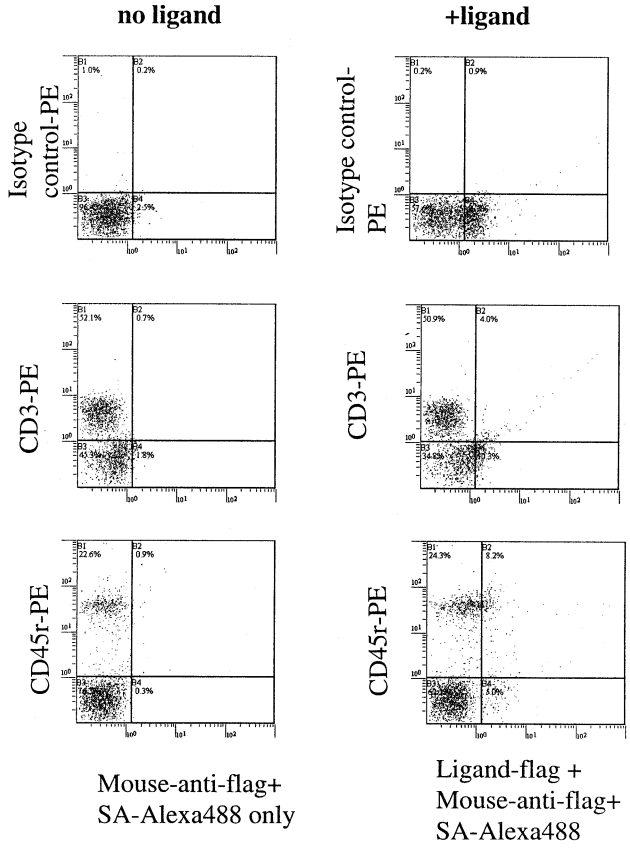

Fig. 6. TWEAK binds normally to murine lymphocyte subsets. Peripheral blood lymphocytes from wild-type mice were incubated for 10 min in the presence or absence of FLAG-tagged murine TWEAK (3 μg). Cells were then incubated with 0.5 μg anti-FLAG conjugated to biotin, following the blocking of murine Fc receptors with normal mouse serum, and 0.4 μg of each phycoerythrin antibody conjugate as indicated. Hamster IgG2A-PE was used as an isotype control.

the cell type specific surface marker anti-CD3 we observed no interaction with T-lymphocytes (Fig. 6). However analysis using anti-CD45r showed that TWEAK is interacting with a subpopulation of B-cells (Fig. 6). Strong interaction with macrophages was also observed, as was weaker binding to granulocytes (data not shown). No differences in the type of TWEAK-binding cells were observed between wild-type and knockout animals (data not shown).

Taken together these observations indicate that TWEAK exhibits a normal binding profile in several different cell types isolated from a knockout animal in which its proposed receptor has been deleted. The data suggest that the TWEAK ligand has a receptor that is not WSL-1/TRAMP, and WSL-1/TRAMP may have as yet unidentified ligand distinct from TWEAK.

4. Discussion

The continuing identification of numerous new genes related to both TNF and the TNF receptor highlights the biological importance of these gene families. The subgroup of receptors that contain a cytoplasmic death domain is of particular interest as a result of their ability to regulate apoptosis and the activation of NF-κB, events of great biological significance. Death domain containing receptors such as the TNF receptor and FAS have all been shown to be involved in the pathology of human disease, therefore the biological role of the WSL-1/TRAMP receptor is also of considerable interest. In particular its predominant expression in cells from the immune system suggests an important function, and deletion of the chromosomal locus containing the WSL-1/TRAMP gene has been associated with neuroblastoma [17].

The determination of the biological function of WSL-1/TRAMP has been significantly hindered by the lack of its cognate ligand, so the report that the novel TNF-related molecule TWEAK could bind to the receptor promised to be an important breakthrough [13]. However there appeared to be several discrepancies between the previously published data on TWEAK and the function and distribution of the WSL-1/TRAMP receptor. For example, Chicheportiche et al. [16] reported preliminary results that TWEAK did not bind WSL-1/TRAMP and Schneider et al. [14] found that Kym-1 cells bound TWEAK but did not express WSL-1/TRAMP.

Analysis of the interaction between WSL-1 and members of the TNF ligand family (including TWEAK) by ELISA, immune precipitation or binding to cells transfected with a WSL-1/TRAMP construct, could not reproduce the in vitro binding previously reported [5]. In a series of experiments utilising both human and murine TWEAK ligand, it has been shown that the TWEAK protein has no effect on experimental cell lines transfected with WSL-1/TRAMP, again in contrast to previously reported results which suggested TWEAK increased WSL-1 mediated apoptosis [5]. The data also demonstrate that TWEAK can bind strongly to cells that do not express WSL-1/TRAMP on the cell surface, suggesting strongly the presence of a different TWEAK receptor on these cells. Biological activity of TWEAK is also observed in these cells, despite the absence of the proposed receptor. Finally, various cell types isolated from mice in which the WSL-1/TRAMP receptor has been deleted have been shown to retain their wild-type ability to interact with the TWEAK ligand.

Taken together, biochemical interaction studies and data obtained with knockout mice strongly suggest that TWEAK is not the ligand for the death domain containing receptor WSL-1/TRAMP. Therefore it is unlikely that WSL-1/TRAMP plays a role in the biological function of TWEAK.

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