Differential Role of TNF Receptors in Cellular Trafficking of Intact TNF

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Key Words

TNF α • Receptor-mediated endocytosis • Intracellular stability • Exocytosis

Abstract

Background/aims: Although ligand signaling and degradation within the cell have received much attention, few studies have quantified the role of receptors on the transcytosis of ligand into and out of the cell in intact form. Accordingly, we determined the differential role of the two receptors for tumor necrosis factor α (TNFR1, TNFR2) on cellular transcytosis. **Methods:** TNFR1 and TNFR2 were overexpressed in HEK293 cells by transient transfection. Cell surface binding, endocytosis, and exocytosis of 125 I-TNF were quantified. Degradation was determined by acid precipitation and size-exclusion chromatography. **Results:** TNFR1-mediated uptake of TNF was faster than TNFR2-mediated uptake of TNF. TNFR2, however, exhibited greater capacity, leading to a higher percentage release of TNF into the exocytosis medium. Rather than being degraded, most of the TNF inside the cell remained intact for 1 h. Both receptors exerted protective roles against degradation, but there was no cooperativity

between them. **Conclusion:** The effects of TNFR1 and TNFR2 in shepherding TNF across the cell illustrate the differential roles of receptors on the cellular trafficking of the ligand in intact form so as to facilitate its biological effects.

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Introduction

It is increasingly recognized that endocytosis of a ligand-receptor complex does not indicate termination of the signal [1]. Rather, endocytosis can be necessary for the complete activation of signaling events [2]. Apart from the general implications of intracellular trafficking, tumor necrosis factor α (TNF) deserves special attention because of its dual effects of either cell death or cytoprotection. TNF binds to TNFR1 (p55 or p60) and TNFR2 (p75 or p80), which show distinctive binding kinetics [3, 4]. These single transmembrane receptors recruit receptor associated proteins for diverse signaling events [5-9]. However, internalization of the TNF-receptor complex and its subsequent fate are not fully understood.

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In studies of mouse blood-brain barrier and cultured cerebral microvessel endothelial cells, we have shown that TNF endocytosed from the apical lumen can exit from the basolateral surface [10-14]. Such transcytosis suggests that the internalized protein ligand remains intact within the cell. Indeed, the cytokine leptin is stable intracellularly for more than an hour after endocytosis [15]. Although we have seen that TNF remains relatively stable in cultured brain endothelial cells, the pathway it takes to avoid degradation is not clear [16]. Inferred from studies of other ligands, there might be several potential routes, including fusion of intra-luminal vesicles with re-targeting of the cargo to exocytotic pathways [17], relay through different receptor subtypes as with the axonal transport of neurotrophin-3 [18, 19], formation of intracellular channels, and calcium- and ATP-mediated quantum release. In this study, we examined the differential involvement of TNFR1 and TNFR2 in the endocytosis, intracellular stability, and exocytosis of TNF.

Materials and Methods

Cell lines and reagents

Human embryonic kidney (HEK) 293 cells, which express low levels of endogenous TNF receptors and exhibit high transfection efficiency, were obtained from American Type Culture Collection (ATCC, Manassas, VA). The cells were grown in DMEM containing 10% fetal bovine serum and antibiotics. The cDNAs for mouse TNFR1 and TNFR2 were kindly provided by Genentech (South San Francisco, CA) and subcloned into the pcDNA3.1 vector. Antibodies against TNFR1, TNFR2, and the internal control β -actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Recombinant mouse TNF α was purchased from R & D Systems (Minneapolis, MN). Radioactive labeling with ^{125}I (Amersham Life Science, Piscataway, NJ) was achieved by the iodogen method (Pierce Biotechnology, Rockford, IL). ^{125}I -TNF was purified by elution on columns of Sephadex G-10 and had a specific activity of 60 – 80 $\mu\text{Ci}/\mu\text{g}$.

To compare the relative expression of TNFR1 and TNFR2, four additional cell lines as well as freshly isolated microvessels were used: RBE4 rat cerebral microvessel cells, hCMEC/D3 human cerebral microvessel endothelial cells, C6 astrogloma, and H19-7 hippocampal neuronal cells. RBE4 and hCMEC/D3 were provided by Dr. Pierre O. Couraud (Institute of Cochin, Paris, France) [20, 21]. C6 and H19-7 cells were obtained from ATCC.

Transfection

Constructs of full-length TNFR1 and TNFR2 in pcDNA3.1 were used to transiently transfect HEK293 cells. When the cells in 6-well plates grew confluent 1 d after seeding, 2 μg of plasmid DNA was introduced into the cells with lipofectamine

2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's recommendations. Triplicates of cells were used, and a control group transfected with pcDNA3.1 empty vector was included.

Western blot

HEK293 cells grown in 6-well plates were transfected with 2 $\mu\text{g}/\text{ml}$ of plasmid DNA at 90% of confluency, and harvested 24 – 30 h after transfection. Whole cell lysate was obtained by incubation of the cells with RIPA buffer containing a protease inhibitor cocktail. Protein concentration in the supernatant was quantified by the BCA assay, and 40 μg of protein was loaded onto each lane of an 8% SDS-polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane. The membrane was blocked with 5% non-fat dry milk, incubated with primary antibodies against TNFR1, TNFR2, or β -actin (Santa Cruz Biotechnology) followed by HRP-conjugated secondary antibody, and then developed with ECL chemiluminescent reagent (Pierce).

Real-time RT-PCR

As described previously [14], a cerebral microvessel-enriched preparation was obtained from the brains of B6 mice by the capillary depletion method and total RNA was extracted. Real-time RT-PCR for mouse TNFR1, TNFR2, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed on an Applied Biosystems 7900 with Taqman primers and probes and a one-step RT-PCR core kit. The relative amount of TNFR1 and TNFR2 mRNA was calculated by subtraction of their cycle threshold (CT) from that of GAPDH, and the value of power ($2^{-\Delta\Delta\text{CT}}$) was obtained for each sample.

Binding of ^{125}I -TNF

Groups of cells in triplicate wells were prepared as indicated in the *Results* section. The cells were studied 24 $\pm$ 6 h after transfection. Relative cell surface binding was determined by incubation of the cells with ^{125}I -TNF (400,000 cpm/ml) for 3h at 4°C. This was preceded by equilibrium of the cells in transport buffer (DMEM with 20 mM of HEPES and 0.5% of bovine serum albumin) for 30min on ice. The unbound ligand was removed by two cold PBS washes, and the specific binding was measured after 20min incubation of the cells in ice-cold acid wash buffer (0.2 N acetic acid, pH 3). The percentage binding compared with the radioactivity added was calculated for each well of cells and normalized to protein concentration determined by the BCA assay.

Endocytosis of ^{125}I -TNF

After pre-equilibrium in transport buffer for 30min at 37°C, the cells were incubated with ^{125}I -TNF constantly present in the pre-warmed transport buffer for 20 – 30min at 37°C. At the end of the study, the unbound ligand was rapidly removed by two washes with ice-cold PBS, and the radioactivity remaining at the cell surface was collected by incubation with ice-cold acid wash buffer. After removal of specific surface binding by this procedure, the cells were lysed, scraped, and collected in test tubes for further degradation assays or direct measurement of radioactivity in a γ -counter. Maximal potential internalization

is the sum of the endocytosed ^{125}I -TNF together with that remaining at the cell surface at the end of the experiment.

Exocytosis assays

The cells were incubated with ^{125}I -TNF (about 400,000 cpm/ml) for 30min at 37°C. After removal of the medium and a PBS wash, the cells were rapidly washed with a mild acid buffer (pH 5.0) three times to remove surface-bound ligand without compromising membrane integrity [22]. Control studies show that this is sufficient to remove cell surface binding [23]. Different groups of cells were then incubated with pre-warmed transport (exocytosis) buffer for 5 - 30min. This exocytosis buffer was collected for acid precipitation or size-exclusion chromatography and measurement of radioactivity, along with cell lysates. The percent ^{125}I -TNF exocytosed was determined by the radioactivity recovered from the exocytosis buffer divided by the total radioactivity from the exocytosis buffer, acid wash, and cell lysate.

Degradation assays

The extent of intracellular degradation of ^{125}I -TNF was determined by acid precipitation and size-exclusion chromatography. For acid precipitation, an equal amount (1 ml) of 30% NaCl-oversaturated trichloroacetic acid was added to the buffer. The vortex-mixed solution was maintained on ice for 15min and centrifuged at 3500g for 15min at 4°C. The precipitate and supernatant were carefully separated and the radioactivity was measured.

Acid precipitation of ^{125}I -TNF in the exocytosed fraction reflects the percentage remaining intact after any intracellular degradation occurring before exocytosis. To better differentiate the pattern of degradation, size-exclusion chromatography was conducted with P10 polyacrylamide gel (Bio-Rad Laboratories, Hercules, CA) packed into 9cm x 6 mm columns. The radioactive substances were eluted by phosphate buffered saline. Forty-five fractions were collected (100 μl /fraction) at a flow rate of 100 μl /min.

Statistical analyses

Means are presented with their standard errors. One-way analysis of variance (ANOVA) was performed to compare the differences among groups, and Tukey's post-hoc test was used when an overall difference was detected. Kinetics studies were analyzed by the least squares method with the Prism GraphPad statistical program (San Diego, CA).

Results

Higher level of TNFR1 expression than TNFR2

HEK293 cells showed a higher level of protein expression of TNFR1 than of TNFR2 at 24 - 30h after transient transfection of equal amounts of the plasmids. Endogenous levels could be detected in naïve cells (lane 1) and cells mock-transfected with the pcDNA3.1 vector (lane 2). Densitometric analysis showed that the increase

Fig. 1. Expression of receptor proteins in HEK293 cells 24-30h after transient transfection was determined by Western blot. Both TNFR1 and TNFR2 showed higher expression than the empty vector-transfected group. Lane 1: HEK293 cells with lipofectamine 2000 only; Lane 2: cells transfected with pcDNA3.1, 2 $\mu\text{g}/\text{ml}$; Lane 3: cells transfected with TNFR1 (upper panel) or TNFR2 (lower panel), 2 $\mu\text{g}/\text{ml}$; Lane 4: cells transfected with both TNFR1 and TNFR2 (1 $\mu\text{g}/\text{ml}$ each). The relative level of expression of TNFR1 and TNFR2 in the total cell lysate was further shown by the equal amount of β -actin, an internal control.

of TNFR1 protein was higher than that of TNFR2 (lane 3). Co-transfection of both TNFR1 and TNFR2 plasmids (each having half of the amount of the single transfection) lowered the level of expression of each (Fig.1).

Greater binding but slower endocytosis by TNFR2 than TNFR1

Receptor binding was determined by incubation of the cells overexpressing the receptors with ^{125}I -TNF at 4°C. Both TNFR1 and TNFR2 caused a significant increase of cell surface binding ($p < 0.005$). TNFR2-overexpressing cells showed more than two-fold higher binding than the TNFR1-overexpressing cells, despite TNFR1 having higher protein expression as shown above. The percent surface binding of TNF in the TNFR2-overexpressing cells was significantly higher than in the other groups ($p < 0.001$) (Fig.2).

Cellular uptake of ^{125}I -TNF was determined after 20min incubation at 37°C. At the end of the study, the percentage of remaining cell surface ^{125}I -TNF was 120-fold higher in the TNFR2 group than in the control and 19-fold higher than in the TNFR1 group. The percentage of internalized ^{125}I -TNF in the TNFR2-overexpressing cells was also 32-fold higher than in the control and 2.8-fold higher than in the TNFR1 group (Fig.3). Thus, TNFR2

Fig. 2. Surface binding of ^{125}I -TNF in transfected HEK293 cells was determined after 3 h incubation at 4 °C, 24 h after transient transfection of mouse TNFR1 (2 $\mu\text{g}/\text{well}$), TNFR2 (2 $\mu\text{g}/\text{well}$), R1+R2 (1 μg each/well), or pcDNA3.1 empty vector (2 $\mu\text{g}/\text{well}$). Overexpression of the receptors significantly increased ^{125}I -TNF surface binding. The increase was highest in cells overexpressing TNFR2. ***: $p < 0.005$ compared with the vector control ($n = 3$ /group) when analyzed by ANOVA and post-hoc Tukey's test.

Fig. 3. Surface binding and endocytosis ^{125}I -TNF was determined in transfected HEK293 cells after 20min of incubation at 37°C. At 24h after transfection, both TNFR1 and TNFR2 mediated a significantly higher percentage of binding and endocytosis of ^{125}I -TNF. The TNFR2 -overexpressing group showed significantly greater endocytosis and remaining cell surface binding than the R1 group. ***: $p < 0.005$ compared with the vector controls for the same fraction ($n = 3$ /group). One-way ANOVA and Tukey's post-hoc test were performed on the data from each fraction.

played a greater role in the endocytosis as well as having a higher binding capacity for TNF.

TNFR1, however, exerted a more rapid effect in internalizing TNF. In TNFR1-overexpressing cells, cell surface binding was only 30% while internalization was 70%. This is in contrast with the TNFR2-overexpressing cells, where 74% of the total ^{125}I -TNF associated with the cells was still retained at the cell surface at 20min. Therefore, TNFR1 induced faster internalization of ^{125}I -TNF than did TNFR2.

Intracellular fate of TNF after endocytosis

The ability of the internalized TNF to be released by exocytosis was determined 30min after initiation of endocytosis, and the amount of cytokine exocytosed intact between 0 - 30min was analyzed by P10 size-exclusion chromatography. More intact ^{125}I -TNF was exocytosed by the vector-only control (44.6%) and TNFR1 overexpressing cells (49.0%) than by the TNFR2 overexpressing cells (36.8%) or cells overexpressing both TNFR1 and TNFR2 (32.2%) (Fig.4). Further analysis

Fig. 4. Exocytosis was most robust in the TNFR2 and R1R2 co-expressing groups. Intact ^{125}I -TNF accounted for 44.6% of radioactivity recovered in the exocytosis buffer in the control group, 49% in the TNFR1 group, 36.8% in the TNFR2 group, and 32.3% in the co-transfected group.

Fig. 5. Intracellular degradation of ^{125}I -TNF was significantly slowed by overexpression of TNFR1 or TNFR2. The percent precipitability at time 30min was 40.7% for the vector control, 68.3% for TNFR1, 64.0% for TNFR2, and 51.7% for the R1+R2 group ($n = 3$ /time point). The slope of the linear regression, analyzed by the least-squares method with the Prism GraphPad software, did not show significant differences among the groups.

was performed on the kinetic data obtained after acid precipitation in groups of cells (triplicate wells /group) studied at 5, 10, 15, 20, and 30min. Because some of the degradation products in the wide second peak from the size-exclusion chromatography remained precipitable, the values from acid precipitation were higher than those estimated chromatographically. TNFR1 and TNFR2 groups had significantly less degradation than the control group ($p < 0.05$). This was most pronounced during the first 20min (Fig.5).

By contrast to the substantial degradation of the exocytosed ^{125}I -TNF, intracellular ^{125}I -TNF in the TNFR2

and co-transfected groups remained largely intact during the entire 60min (30min of internalization followed by 30min of exocytosis) (Fig.6). The TNFR1 and control groups had relatively lower amounts of total radioactivity (Fig.6, inset). It seems that the control group (pcDNA3.1 transfected cells) showed the most pronounced intracellular degradation, only 56.6% remaining intact at the end of the study (Table 1). However, this might be obscured by the combination of a low value for the reading together with a high background, resulting in overestimation of the extent of degradation. Regardless, the results lead to the conclusion that intracellular

Fig. 6. The amount of radioactivity present in the lysis buffer was more than 15-times higher in the TNFR2 and R1R2 groups than in TNFR1 group which was already 3 times higher than the control. Most of the radioactivity inside the cells after receptor-mediated endocytosis remained as intact ¹²⁵I-TNF.

% intact	control	R1	R2	R1R2
in lysis buffer	56.6	83.6	92.2	92.5
in exocytosis buffer	44.6	49.0	36.8	32.3

Table 1. Degradation of internalized TNF at 60 min.

degradation was not receptor-dependent, although the receptors exerted a protective effect against degradation of TNF.

Once TNF was internalized, its rate of efflux also differed among the groups. Overall, the TNFR2-overexpressing cells showed a larger fraction of radioactivity recovered in the exocytosis medium at all the time points examined. But the greater effect of TNFR2 was mainly present at the internalization and trafficking processes without direct involvement of exocytosis. This is shown by calculation of the percentage of exocytosed TNF as the numerator in relation to the total amount already internalized as the denominator. By this analysis, the efflux rate was slowest in TNFR2-overexpressing cells (0.16 ± 0.01 %/min), followed by TNFR1-expressing cells (0.28 ± 0.05 %/min), both values being significantly less ($p < 0.001$) than the vector control group (0.58 ± 0.05 %/min). Consistent with the observation that TNFR2-overexpressing cells retained more ¹²⁵I-TNF than TNFR1-overexpressing cells, the efflux in the TNFR2

group was significantly slower than in the TNFR1 group ($p < 0.05$) (Fig.7).

Since the TNFR2 group had the slowest efflux rate, the question arises whether the exocytosis might have occurred by a saturable process that was obscured by a surplus of TNF. Whereas addition of excess unlabeled TNF to the endocytosis medium significantly reduced the fraction of ¹²⁵I-TNF exiting the cells in all groups, excess unlabeled TNF α in the efflux medium showed no significant effect. Together, the results show that only TNFR2 had a significant net effect on increasing the efflux of endocytosed TNF α from HEK293 cells. The rate-limiting step occurred during endocytosis, rather than exocytosis.

Relation of overexpression to the in vivo situation

In mice, the levels of expression and relative distribution of TNFR1 and TNFR2 vary among cells. Epithelial and endothelial cells are polarized, showing lateralized distribution of the receptors with more at the

Fig. 7. Time-course of exocytosis of ^{125}I -TNF expressed as the percentage of total ^{125}I -TNF endocytosed, which is the value at time 0 (initiation of exocytosis). Cells overexpressing R1 or R2 showed a significantly ($p < 0.001$) slower efflux rate of internalized ^{125}I -TNF α when compared with the vector-transfected control. The slope of the linear regression was analyzed by the least-squares method with the Prism GraphPad software.

Fig. 8. Level of receptor protein expression in cerebral microvessels and cell lines by Western blot analysis, normalized by the internal control β -actin. (A) Duplicated samples from B6 mouse cerebral microvessels showed that TNFR2 expression was higher than that of TNFR1, but the difference in densitometry analysis was not statistically significant. (B) TNFR1 showed abundant expression in hCMEC/D3 human cerebral microvessel cells, higher than TNFR2. In other cell lines tested, neither receptor showed a high level of expression.

apical surface than at the basolateral surface. However, when grown as monolayers on solid support, these cells become partially depolarized, resulting in underestimation of exocytosis. As there are no ideal cell lines currently available to address BBB transport *in vitro*, we used HEK293 cells which have a high efficiency of transfection. The overexpression studies showed lack of cooperative interaction between TNFR1 and TNFR2. To determine whether the relative distribution of receptor subtypes reflects that occurring in cerebral microvessel endothelial cells, we further determined the expression of TNFR1 and TNFR2 in tissue and non-transfected cells. Normalized to the house-keeping gene GAPDH, the

mRNA for TNFR2 was 1.6 times higher than the mRNA for TNFR1 in microvessels isolated from mouse cerebral cortex, but the difference was not significant. Normalized to the house keeping gene product β -actin, the protein of TNFR2 was 1.1 times greater than that of TNFR1 also without statistical significance (Fig.8A). The level of expression of TNFR1 and TNFR2 varied greatly in the different cell lines. Antibodies against mouse epitopes that cross-react with rat and human TNFRs showed that the expression of TNFR1 was low in RBE4 rat cerebral endothelia, C6 astroglia, and H19-7 neuronal cells. By contrast, the immunoreactivity of TNFR1 in hCMEC/D3 human cerebral endothelia was robust. TNFR2 paralleled

Fig. 9. Diagram of the relative contribution of TNFR1 and TNFR2 in cell surface binding, endocytosis, and exocytosis of ^{125}I -TNF. Overall, TNFR2 mediated a higher percentage exocytosis of intact TNF.

TNFR1 in these cell lines but to a lesser extent (Fig.8B).

Discussion

TNFR1 and TNFR2 show differential distribution and function both in tissue and in the single cell. Most studies have focused on the signaling events mediated by associated proteins. Little attention has been given to the relative roles of these two receptors in enabling TNF to cross the cell in intact form so as to exert its biological effects. We therefore quantified the surface binding, endocytosis, and exocytosis of TNF in cells overexpressing its receptors.

In the overexpression studies on HEK293 cells, protein expression for TNFR1 was higher than for TNFR2. However, radiotracer uptake assays showed that the cell surface binding and endocytosis capacity was higher for TNFR2 than for TNFR1. Despite the greater cell surface distribution of TNFR2, as shown previously

[24], TNFR1 mediated faster endocytosis of TNF. Therefore, we further tested whether the different receptor subtypes mediate different patterns of intracellular degradation of TNF, and whether they cooperate with each other.

Contrary to most conventional expectations, TNF remained intact for a prolonged period (at least 1h) after internalization, and exhibited substantial exocytosis in intact form. The TNF receptors caused significant stabilization of TNF inside the cell, thereby facilitating the release of intact TNF into the exocytosis buffer. Similar findings of prolonged intracellular stability have been reported for leptin transport in overexpression cell models [15]. Overall, TNFR2 caused a greater release of intact TNF, but tended to retain intact TNF intracellularly. Neither receptor showed direct effects on the exocytosis process. Moreover, there was no cooperativity between the receptors.

There are several inherent limitations in overexpression studies with HEK293 cells. Although

these cells provide high transfection efficiency resulting in high levels of TNFR1 and TNFR2 expression, the relative levels of the receptor subtypes may not be the same as in the *in vivo* situation. The lack of full polarization also makes it difficult to study vectorial transport, and the expression and distribution of transport accessory molecules, such as caveolae, chaperon proteins, and cytoskeletal structures, may differ from other epithelial and parenchymal cells. Although efforts continue to generate ideal models for the study of cellular transport, studies with HEK293 cells still provide insights otherwise unavailable from the *in vivo* studies.

We compared the relative expression of TNFR1 and TNFR2 in cerebral microvessels and in cell lines of brain origin. In cerebral microvessels isolated from mouse brain, the mRNA for TNFR2 was actually higher than that for TNFR1 although it did not reach statistical significance. This appears to be consistent with what has been reported in rat brain capillaries [25]. The level of protein expression also was higher for TNFR2 than for TNFR1 in microvessels, as seen in figure 8A. Immortalization of the cells, however, modified this relative relationship of the receptor subtypes. This was seen in both endothelia and brain parenchymal cells (Fig. 8B), warranting caution in interpretation of the findings from any cellular model.

Nonetheless, the results from the overexpression studies showed distinctive roles for TNFR1 and TNFR2 in TNF trafficking. A summary of the data from binding, cellular uptake and exocytosis reveals that 32% of the TNF was bound to TNFR1 whereas 68% was bound to TNFR2 at the cell surface at time 0. At 20 min, 70% of the TNFR1-bound TNF was endocytosed, and this

constituted 22.4% of the total pool of TNF; 26% of the TNFR2-bound TNF was endocytosed (17.7 % of the total pool). After 30min of endocytosis and 30min of exocytosis, 20% of the internalized TNF was recovered in the medium in the TNFR1 group, accounting for more than 4.5% of the internalized TNF. As 49% of the radioactivity in the exocytosis medium was intact TNF, we can conclude that more than 2.2% intact TNF could be released by HEK293 cells transfected with TNFR1. Similarly, in the TNFR2 group, 10% of the internalized TNF was recovered in the exocytosis medium, 37% being intact, and this yielded more than 6.5% intact TNF. This is illustrated in a flow chart as figure 9, which shows a greater role for TNFR2 than TNFR1 in the transcytosis of TNF.

It might have been anticipated that the transport of intact ligand across cells would be an unusual phenomenon, limited to specific epithelial cells such as the endothelia composing the blood-brain barrier, as well as epithelia in intestinal mucosa and renal tubules. Our results, however, indicate that transcytosis of intact ligand after receptor-mediated endocytosis is more universal. This represents an advantage of the use of HEK293 cells. The specific roles of receptors in the intracellular trafficking process, illustrated here with TNF, may have broad implications for biological activity.

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References

- 1 Di Fiore P, De Camilli P: Endocytosis and signaling: an inseparable partnership. *Cell* 2001;106:1-4.
- 2 McPherson PS, Kay BK, Hussain NK: Signaling on the endocytic pathway. *Traffic* 2001;2:375-384.
- 3 Baglioni C, McCandless S, Tavernier J, Fiers W: Binding of human tumor necrosis factor to high affinity receptors on HeLa and lymphoblastoid cells sensitive to growth inhibition. *J Biol Chem* 1985;260:13395-13397.
- 4 Yoshie O, Tada K, Ishida N: Binding and crosslinking of ¹²⁵I-labeled recombinant human tumor necrosis factor to cell surface receptors. *J Biochem (Tokyo)* 1986;100:531-541.
- 5 Lehmann V, Droge W: Demonstration of membrane receptors for human natural and recombinant ¹²⁵I-labeled tumor necrosis factor on HeLa cell clones and their role in tumor cell sensitivity. *Eur J Biochem* 1986;158:1-5.
- 6 Kull FC, Jacobs S, Cuatrecasas P: Cellular receptor for ¹²⁵I-labeled tumor necrosis factor: specific binding, affinity labeling, and relationship to sensitivity. *Proc Natl Acad Sci USA* 1985;82:5756-5760.
- 7 Smith RA, Kirstein M, Fiers W, Baglioni C: Species specificity of human and murine tumor necrosis factor: A comparative study of tumor necrosis factor receptors. *J Biol Chem* 1986;261:14871-14874.

- 8 Lewis M, Tartaglia LA, Lee A, Bennett GL, Rice GC, Wong GH, Chen EY, Goeddel DV: Cloning and expression of cDNAs for two distinct murine tumor necrosis factor receptors demonstrate one receptor is species specific. *Proc Natl Acad Sci USA* 1991;88:2830-2834.
- 9 Goodwin RG, Anderson D, Jerzy R, Davis T, Brannan CI, Copeland NG, Jenkins NA, Smith CA: Molecular cloning and expression of the type 1 and type 2 murine receptors for tumor necrosis factor. *Mol Cell Biol* 1991;11:3020-3026.
- 10 Gutierrez EG, Banks WA, Kastin AJ: Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. *J Neuroimmunol* 1993;47:169-176.
- 11 Pan W, Zadina JE, Harlan RE, Weber JT, Banks WA, Kastin AJ: Tumor necrosis factor α : a neuromodulator in the CNS. *Neurosci Biobehav Rev* 1997;21:603-613.
- 12 Pan W, Kastin AJ: TNF α transport across the blood-brain barrier is abolished in receptor knockout mice. *Exp Neurol* 2002;174:193-200.
- 13 Pan W, Kastin AJ, McLay RN, Rigai T, Pick CG: Increased hippocampal uptake of TNF α and behavioral changes in mice. *Exp Brain Res* 2003;149:195-199.
- 14 Pan W, Ding Y, Yu Y, Ohtaki H, Nakamachi T, Kastin AJ: Stroke upregulates TNF α transport across the blood-brain barrier. *Exp Neurol* 2006;198:222-233.
- 15 Tu H, Pan W, Feucht L, Kastin AJ: Convergent trafficking pattern of leptin after endocytosis mediated by ObRa – ObRd. *J Cell Physiol* 2007;212:215-222.
- 16 Pan W, Kastin AJ, Daniel J, Yu C, Basbaum A, Baryshnikova LM, von Bartheld CS: TNF trafficking in cerebral vascular endothelial cells. *J Neuroimmunol* 2007;185:47-56.
- 17 van der Goot FG, Gruenberg J: Intra-endosomal membrane traffic. *Trends Cell Biol* 2006;16:514-521.
- 18 Wang X, Butowt R, Vasko MR, von Bartheld CS: Mechanisms of the release of anterogradely transported neurotrophin-3 from axon terminals. *J Neurosci* 2002; 2:931-945.
- 19 Butowt R, von Bartheld CS: Sorting of internalized neurotrophins into an endocytic transcytosis pathway via the Golgi system: ultrastructural analysis in retinal ganglion cells. *J Neurosci* 2001;21:8915-8930.
- 20 Pan W, Yu Y, Cain CM, Nyberg F, Couraud P-O, Kastin AJ: Permeation of growth hormone across the blood-brain barrier. *Endocrinol* 2005;146:4898-4904.
- 21 Weksler B, Sublieau E, Perriere N, Charneau P, Holloway K, Leveque M, Tricoire-Leignel H, Nicotra A, Bourdoulous S, Turowski P, Male D, Roux F, Greenwood J, Romero I, Couraud P: Blood-brain barrier-specific properties of a human adult brain endothelial cell line. *FASEB J* 2005;19:1872-1874.
- 22 McGraw TE, Subtil A: Protein trafficking; *Current Protocols in Cell Biology*: John Wiley & Sons, Inc., 1999, pp 15.3.1-15.3.23.
- 23 Tu H, Kastin AJ, Pan W: CRH-R1 and CRH-R2 are both trafficking and signaling receptors for urocortin. *Mol Endocrinol* 2007;21:700-711.
- 24 Bradley JR, Thiru S, Pober JS: Disparate localization of 55-kd and 75-kd tumor necrosis factor receptors in human endothelial cells. *Am J Pathol* 1995;146:27-32.
- 25 Osburg B, Peiser C, Dömling D, Schomburg L, Ko YT, Viogt K, Bickel U: Effect of endotoxin on expression of TNF receptors and transport of TNF- α at the blood-brain barrier of the rat. *Am J Physiol* 2002;283:E899-E908.