Published in final edited form as:

J Neuroimmunol. 2007 April ; 185(1-2): 47–56.

TNF α trafficking in cerebral vascular endothelial cells

Weihong Pan^{1,*}, Abba J. Kastin¹, Jeremy Daniel¹, Chuanhui Yu¹, Larisa M. Baryshnikova², and Christopher S. von Bartheld²

¹Pennington Biomedical Research Center, Baton Rouge, Louisiana, USA

²Departments of Physiology & Cell Biology, University of Nevada, Reno, Nevada, USA

Abstract

Using small tags, we tracked the pathway of tumor necrosis factor (TNF) α across cerebral vascular endothelial cells. In cerebral microvessel derived RBE4 cells, ¹²⁵I-TNF α had rapid endocytosis within the first 20 min and showed substantial exocytosis in the intact form. Biotinylated TNF α was detected at different time points after endocytosis by streptavidin-Quantum dots which showed its time-dependent colocalization with intracellular organelles. In mice, electron microscopic autoradiography after intravenous injection of ¹²⁵I-TNF α showed its transcytosis, as signals emerged on the abluminal side of the endothelial cells and reached brain parenchyma. The vesicular trafficking of TNF α reflects the immunomodulatory potential of peripheral cytokines for the CNS.

Keywords

Endothelium; Blood-brain barrier; Endocytosis; Transport; TNF α ; Cytokine

1. INTRODUCTION

Despite the important influence of the proinflammatory cytokine tumor necrosis factor α (TNF α) on the blood-brain barrier (BBB), trafficking of the ligand TNF α in the cerebral microvessel endothelial cells composing the BBB has received less attention than its receptors. Transcytosis of an exogenous peptide or protein ligand from the apical to basolateral side of a cell does not seem to be a universal phenomenon but can exert important physiological functions. Microvessel endothelial cells composing the BBB are polarized, being joined by tight junctions, underlined by a continuous basement membrane, and reinforced by pericytes and astrocytic endfeet. These endothelial cells have lateralized distribution of intracellular enzymes and transporter proteins in the luminal (apical) and abluminal (basolateral) membranes. This makes specific transport systems for selective cytokines particularly important in facilitating transmission of peripheral inflammatory and immune signals to the CNS by way of the BBB.

Since the BBB constitutes the largest neurovascular interface, selective transport systems play significant roles along with neuroendocrine feedback, axonal transport, and direct effects of blood-borne signals on circumventricular organs (CVOs). The CVOs are regions where

*Corresponding author: Weihong Pan, MD, PhD, Pennington Biomedical Research Center, 6400 Perkins Road, Baton Rouge, LA 70808, USA, Tel. 1-225-763-2707, Fax 1-225-763-0261, e-mail: weihong.pan@pbrc.edu

Grant support is provided by NIH (NS45751 and NS46528 to WP, DK54880 to AJK, and EY12841 to CSvB).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

circulating cytokines can exert direct effects. However, such extracellular pathways accounts for less than 5% of the brain uptake of blood-borne cytokines such as interleukin-1 α (Plotkin et al. 1996), and there is an ependymal layer with intercellular tight junctions between the CVOs and rest of the brain that constitutes an intact barrier (Johanson 1995).

So far, the most definitive evidence for the direct route of a proinflammatory cytokine to the CNS derives from studies with interleukin-1 α (Banks et al. 1991,2001). After intravenous delivery, interleukin-1 β reaches the posterior division of the septum in mice and impairs memory. For TNF α , transport across the BBB is speculated to contribute to the CNS effects of inflammatory and immune signals from the peripheral circulation (Gutierrez et al. 1993). This transport is probably involved in the neuromodulatory functions of TNF α in such processes as sleep, feeding, fever, sickness behavior resulting from peripheral inflammation, and neuroregeneration (Pan et al. 1997c).

In experiments both in vivo and with Transwell culture, we have shown that TNF α transport is mediated by its receptors. Transport is decreased in knockout mice lacking either of the two TNF α receptors (TNFR1: p55, TNFR2: p75), abolished in the double-receptor knockout mice (both p55 and p75), and upregulated in an autoimmune disorder and several forms of CNS trauma and ischemia (Pan et al. 1996,1997a;2003b,2006;Pan and Kastin 2002). Further understanding of the intracellular trafficking process has potential therapeutic benefit for interventions involving the transport system of TNF α in these disorders.

In fibroblast L-929 cells, Mosselmans et al. showed that colloid gold-labeled TNF α was endocytosed in clathrin-coated pits, accumulating in endosomes and multivesicular bodies, and eventually degraded in lysosomes (Mosselmans et al. 1988). Yet, it is uncertain whether gold labeling alters the trafficking route of TNF α . There also is evidence of vesicular endocytosis of membrane-associated 26 kD endogenous TNF α in mouse macrophages (Shurety et al. 2001). Most reports, however, address the trafficking of the TNF α receptor rather than of TNF α itself, and these involve non-BBB cells (Ding et al. 1989;Vuk-Pavlovic and Kovach 1989;Bradley et al. 1993;Higuchi and Aggarwal 1994;Ledgerwood et al. 1998;Schütze et al. 1999;Schneider-Brachert et al. 2004).

We therefore focused on devising more accurate methods for morphological detection of TNF α trafficking by use of smaller tags that are less likely to alter the trafficking and degradation route of the ligand studied, and characterizing the kinetics of binding, endocytosis, and exocytosis of TNF α in cultured cerebral microvessel endothelial cells. Although the existing BBB cell lines have limitations of partial depolarization and limited tight junction formation in culture, they can be used to examine the mechanisms of cytokine endocytosis, intracellular trafficking, and the exocytotic processes occurring during BBB transport in vivo. Thus, the results would reflect novel aspects of the dynamic interactions of TNF α with the BBB.

2. MATERIALS AND METHODS

Cell lines and reagents

RBE4 rat brain microvessel endothelial cells were generously provided by Dr. Pierre Olivier-Couraud of the Institute of Cochin and Neurotech in Paris. Antibodies against TNFR1, TNFR2, and clathrin heavy chain were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody against caveolin-1 was purchased from BD Biosciences (Palo Alto, CA). Antibodies against early endosome antigen 1 (EEA1) and β -coactomer protein (β COP, marking the Golgi complex) were from Affinity Bioreagents (Golden, CO). Cell culture and treatment reagents were from Sigma (St. Louis, MO). Reagents for electron microscopic (EM) histology were purchased from Electron Microscopy Sciences (Hatfield, PA).

Bioconjugation of TNF α

Recombinant mouse TNF α was purchased from R & D Systems (Minneapolis, MN). Radioactive labeling (radiolabeling) with ^{125}I (Amersham Life Science, Piscataway, NJ) was achieved by the iodogen method (Pierce Biotechnology, Rockford, IL). ^{125}I -TNF α was purified by elution on columns of Sephadex G-10 and had a specific activity of 60 – 80 $\mu\text{Ci}/\mu\text{g}$. Biotinylation was performed by incubation of 1mg/ml of TNF α with 20 mg/ml of freshly dissolved sulfo-NHS-biotin (Pierce) at 4 °C for 2 h. The reaction mixture was dialyzed against phosphate-buffered saline (PBS) at 4°C overnight. The concentration of biotinylated TNF α was determined with a bicinchoninic acid (BCA) protein assay kit (Pierce), and 5 ng/ml was used for the endocytosis studies.

Binding and endocytosis of ^{125}I -TNF α

The treatment groups consisted of triplicates of RBE4 cells grown confluent in 6-well plates coated with rat tail collagen type I. Two types of experiments were conducted. In the pulse-chase studies, the cells were incubated with ^{125}I -TNF α for 2 h at 4° C before removal of unbound ^{125}I -TNF α and initiation of endocytosis at 37°C. In the uptake assays, the cells were incubated directly with ^{125}I -TNF α constantly present in the transport buffer at 37° C. In kinetic studies, a control group at 0 time was included; this group of cells was kept on ice without chasing at 37°C. In selected cases, transport buffer containing more than a 200-fold excess of unlabeled TNF α was included to determine the extent of nonspecific binding and to test saturability of the trafficking process.

Thirty min before the experiment, the cells were equilibrated in transport buffer at 37°C. The transport buffer for RBE4 cells was HAM's F10 and MEM α medium containing 25 mM HEPES and 0.5% albumin. ^{125}I -TNF α ($430,000 \pm 20,000$ cpm/ml) was added at time 0, and the plates were placed in a 37°C shaking water bath until the end of the experiment (0 – 90 min), at which time they were transferred to ice and the transport buffer was rapidly replaced by ice-cold PBS. The 0 min group was kept on ice throughout. A mild acid wash was performed with 0.2 N acetic acid, pH 3.5. 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 was the sum of the endocytosed ^{125}I -TNF α together with that remaining at the cell surface at the end of the experiment.

Exocytosis assays

The cells were incubated with ^{125}I -TNF α (about 400,000 cpm/ml) for 30 min at 37°C. After removal of the medium and a PBS wash, the cells were washed with a mild acid buffer (pH 5.0) and a quick PBS rinse to remove surface-bound ligand without compromising membrane integrity (McGraw and Subtil 1999). Afterwards fresh exocytosis buffer (same as the transport buffer used for endocytosis assays) was added and the cells were incubated for 2.5 - 60 min. This exocytosis buffer was collected for acid precipitation and measurement of radioactivity, along with cell lysates. The percent ^{125}I -TNF α 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. Acid precipitation of ^{125}I -TNF α in the exocytosed fraction reflects the percentage remaining intact after the intracellular degradation occurring before its exocytosis. For acid precipitation, an equal amount (1 ml) of 30% NaCl-oversaturated trichloroacetic acid is added to the buffer. The vortex-mixed solution was maintained on ice for 15 min and centrifuged at 3500 g for 15 min at 4 °C. The precipitate and supernatant were carefully separated and the radioactivity measured.

High performance liquid chromatography (HPLC)

Reversed phase HPLC was performed to determine the eluting position of intact ^{125}I -TNF α and extent of degradation in cellular supernatant at different time points after endocytosis or exocytosis. About 40,000 cpm of sample was subjected to HPLC, and separated on a C4 protein column by a linear gradient, with the mobile phase being acetonitrile which increased from 0% to 70% over 30 min. One ml fractions were obtained for measurement of the radioactivity.

Immunofluorescent staining and confocal microscopy

RBE4 cells were grown on collagen-coated coverslips until about 80% confluency. The cells were incubated with TNF α (5 - 10 ng/ml) or medium only (control) for 2 - 10 min at 37 °C to induce endocytosis. Afterwards the cells were rapidly rinsed with ice-cold PBS, fixed with paraformaldehyde, and permeabilized with Triton X-100, as described above. After blocking of nonspecific binding, the cells were incubated with primary antibodies against caveolin-1, TNFR1, or TNFR2 for 2 h at room temperature. After thorough wash with PBS, the cells were incubated with Alexa488-conjugated secondary antibody against caveolin-1 and biotinylated secondary antibody against TNFR1 or TNFR2 for 1 h at room temperature. The cells were then washed and incubated with streptavidin-conjugated Qdot-605 for 30 min at room temperature, washed, and mounted on slides. Colocalization of caveolin-1 with TNFR1 or TNFR2 was analyzed with a Zeiss Meta 510 confocal microscope.

Intracellular trafficking of biotinylated TNF α

Biotinylated TNF α (5 ng/ml) was added to RBE4 cells grown on coverslips at 37 °C and allowed to endocytose for the desired time periods. At the end of the experiment, the cells were quickly rinsed with ice-cold PBS, and fixed with 3% paraformaldehyde at 4 °C for 20 min. After permeabilization with 0.2% Triton X-100 and blockade of nonspecific binding with 10% normal serum, the cells were incubated with a primary antibody against membrane microdomains or organelles, as described below, for 2 h at room temperature. The cells were then incubated with an Alexa488 or Alexa594-conjugated secondary antibody along with streptavidin-conjugated Qdot (Quantum Dot, now Invitrogen, Carlsbad, CA) -605 (for colocalization with Alexa488 dye) or -525 (for colocalization with Alexa594). Potential colocalization of biotinylated TNF α -streptavidin Qdot 605 with Alexa488-labeled organelles was analyzed with a Zeiss Meta510 confocal microscopy. For antibodies against organelle markers, which were incubated with secondary antibodies conjugated to Qdot-525 after blocking with biotin solution, colocalization of the two types of Qdots with different emission spectra was also performed by laser confocal microscopy. Negative control groups to verify specificity of the fluorescence included incubation of the cells with Qdot only, secondary antibody only, and the use of blocking peptides for TNFR1 and TNFR2 (obtained from Santa Cruz Biotechnology). To confirm specific endocytosis of biotinylated TNF α , additional groups with inclusion of 200-fold excess of unlabeled exogenous TNF α were also tested to abolish the vesicular distribution of biotinylated TNF α .

EM autoradiography

In accordance with a protocol approved by the Institutional Animal Care and Use Committee, C57 mice (5 - 7 week old) were anesthetized by intramuscular injection of ketamine and xylazine, and ^{125}I -TNF α (4 μCi /mouse in 100 μl of lactated Ringers solution containing 1% albumin) was delivered in a bolus into the isolated left jugular vein. Thirty min later, mice were perfused intracardially with 30 ml of saline, followed by 60 ml of 1% of paraformaldehyde (PFA) and 2% of glutaraldehyde (GA) in PBS at a perfusion rate of 1 ml /min. The brains were post-fixed in 0.5 % PFA /2.5% GA in PBS for at least 2 h at 4°C, and 200 μm coronal sections through the cerebral cortex were obtained by vibratome sectioning. The sections were further fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 2 h, dehydrated with

gradient ethanol, and dissected into 2 x 2 mm² blocks. The tissue blocks were vigorously incubated in 33%, 67%, and then 100% propylene oxide overnight. Afterwards, the blocks were infiltrated with Epon 812 resin mixture, embedded at 60 °C for 72 h, and counted in a γ -counter. Tissue blocks containing 100 – 250 cpm were transferred to the University of Nevada for further processing and analysis.

Ultrathin sections of 80 nm thickness were collected on 200M mesh copper grids, coated with a monolayer of L4 (Liford) emulsion, and exposed in the dark for 3 m (von Bartheld 2001, 2004). The emulsion-coated grids were developed with D19 (Kodak), fixed, and lightly stained with lead citrate. Areas of interest (blood vessels /capillaries) were identified at 1,100 x magnification and images were captured digitally at 10,000 – 25,000 x magnification on a Philips CM10 transmission electron microscope equipped with a Gatan digital system (792 BioScan). Images of 17 sections from 3 blocks and two animals were analyzed at a final magnification of 20,000 – 50,000 x. Tissues from control groups included mice without iv injection of ¹²⁵I-TNF α , and mice with 200-fold excess of unlabeled TNF α in addition to ¹²⁵I-TNF α , neither of which showed significant accumulation of silver grains.

Statistical analyses

Means are presented with their standard errors. One-way analysis of variance was performed to compare the differences among groups, and Tukey's post-hoc test was used when an overall difference was detected.

3. RESULTS

1. Kinetics of ¹²⁵I-TNF α binding and endocytosis in RBE4 cells

After binding at 4°C for 2 h, the surface-bound ¹²⁵I-TNF α was allowed to internalize for various intervals between 0 – 90 min. Endocytosis at 37°C showed a time-dependent increase within the first 30 min that correlated with a significant reduction of surface binding ($p < 0.005$, inverse linear correlation). For surface binding, the dissociation constant was calculated to be 0.16 ± 0.01 and the half-life 4.38 ± 0.02 min (Fig.1A). When endocytosis occurred from a continuous pool of ¹²⁵I-TNF α present in the medium, the maximal potential internalization also showed a significant increase by 60 min, suggesting either upregulation of the internalization complex or re-entry of exocytosed ¹²⁵I-TNF α at this time (Fig.1B). Both binding and internalization of ¹²⁵I-TNF α were saturable processes, as shown by the significant inhibitory effects of excess unlabeled TNF α (Fig.1C). The small fraction of persistent binding and endocytosis of ¹²⁵I-TNF α in the presence of excess unlabeled TNF α probably reflects constitutive receptor recycling related to bulk flow of the membrane, which is non-saturable and independent of ligand binding.

2. Dynamic changes of receptors and membrane microdomains after TNF α stimulation

We have previously shown that the transport of TNF α across mouse brain microvessel endothelial cells is mediated by both TNFR1 and TNFR2 (Pan and Kastin 2002). In the basal state in RBE4 cells, TNFR1 showed a predominant localization in the Golgi complex whereas TNFR2 did not (Fig.2A). This is consistent with observations in other cell types that R1 is present in the Golgi complex whereas R2 is a cell-surface receptor (Jones et al. 1999). There is some controversy concerning the evidence that caveolae-mediated endocytosis probably leads to transcellular transport rather than the intracellular degradation seen with clathrin-mediated endocytosis (Milici et al. 1987; Ghitescu.L. and Bendayan 1992; Schnitzer 2001). Therefore, we further determined the interactions between the receptors and caveolin-1 upon TNF α binding. Western blot showed that RBE4 cells express caveolin-1. Confocal analysis of immunofluorescence showed that neither receptor was colocalized with caveolin-1 under basal conditions; however, stimulation by TNF α at 37 °C for 2 min resulted in increased

colocalization of TNFR1 and TNFR2 with caveolin-1 (Fig.2B). Caveolin-1 is a major component of membrane invagination caveolae, although intracellular localization in organelles such as the Golgi complex is also evident (Bradley et al. 1993). The ligand-induced colocalization of TNF α receptors with caveolin-1 is consistent with electron microscopic evidence of colocalization of TNF α with caveolae, as seen in L929 cells (Mosselmans et al. 1988).

3. Intracellular trafficking of biotinylated TNF α

To differentiate the endocytosed TNF α from endogenous TNF α , which might have been induced by TNF α treatment, we labeled the exogenous TNF α with biotin so that it could be identified after selective interaction with streptavidin-conjugated Qdot. As seen in figure 3A, endocytosed TNF α showed a vesicular pattern of distribution over a time course of 2 – 20 min. Three negative control groups were included: cells with internalized biotinylated TNF α without incubation with streptavidin-Qdot, cells with internalized biotin and the same staining procedure, and cells without internalization but incubated with streptavidin-Qdot. Since biotin is a vitamin rapidly transported across the BBB, the biotin-only control excluded the possibility that the observed fluorescence represented dissociated biotin itself. None of the controls showed fluorescent staining and at none of the times examined (2, 5, 10, 20, and 30 min) did biotinylated TNF α co-localize with the β COP marker of the Golgi complex. Similarly, there was no significant colocalization of endocytosed biotinylated TNF α with lamp1 (marker for lysosomes) or 20s protein (marker for proteasomes) at the times examined. Figure 3B shows representative images at 20 min.

4. TNF α internalized into RBE4 cells can exit in intact form

To test whether endocytosed TNF α could exit the cells in intact form, RBE4 cells were loaded with 125 I-TNF α for 20 min. Subsequently, the amount of radioactivity released into fresh medium was measured, and the percentage of acid precipitable radioactivity calculated. About 11% of the endocytosed 125 I-TNF α was exocytosed by 30 min, 85% of which was acid precipitable. Taking into consideration that 2% of the surface-bound 125 I-TNF α was internalized, about 0.2% of the internalized TNF α could be transcytosed in intact form, and the number would be higher if there were a continuous source of TNF α for endocytosis. Degradation and dissociation of free iodine mainly occurred at later times (20 – 60 min) after initiation of exocytosis, and prolongation of the incubation time to 60 min did not further significantly increase the percent exocytosed (Fig.4A). Further analysis by HPLC showed that more than 50 % of the radioactivity recovered in the extracellular buffer 30 min after initiation of exocytosis represented intact TNF α (Fig.4B).

5. EM autoradiographic demonstration of TNF α transcytosis

Internalization of TNF α has been shown previously at the ultrastructural level with colloidal-gold-tracing in a fibroblast cell-line in vitro (Mosselmans et al. 1988), but little is known about the route of TNF α when it transcytoses the BBB in vivo (Gutierrez et al. 1993). Using qualitative and quantitative autoradiography at the ultrastructural level, we showed that 30 min after iv injection, silver grain signals representing radiolabeled TNF α accumulated in and around blood vessels of cerebral cortex (Fig.5). Most of the signals were seen in close vicinity to blood vessels (within 15 μ m from the vessel wall), whereas the rest of the brain parenchyma contained background levels of silver grains (less than 5% of the grain density seen around blood vessels, equivalent to about 1-2 silver grains per 256 μ m²). The distribution of silver grains (n = 320) in and around blood vessels was quantified from 127 images (taken from 3 tissue blocks and 2 animals). As shown in table 1, the largest fraction (about 35%) of silver grains was localized over endothelial cells (including their membranes), while 25% was in the vessel lumen, and more than one third (36%) had transcytosed across the BBB into the brain

parenchyma, including astrocytes, neurons, and the neuropil. Grains over presumptive pericytes and smooth muscle cells were rare, each accounting for only 1.6% of all grains analyzed (Table 1).

Typical examples of the localization of TNF α are shown in Fig.5A and 5B. Silver grains show TNF α distributed in the blood vessel lumen, endothelial cells (E1), pericytes (P1), smooth muscle cells (M1) and surrounding brain parenchyma. Silver grains in the parenchyma presumably represent TNF α which completely crossed the BBB. As expected for the endocytotic pathway, TNF α was associated with coated vesicles (CV), non-coated vesicles (V), early endosomes (EE), and endoplasmic reticulum (ER) during its transit in endothelial cells. Detailed analysis of 17 tissue sections showed that multivesicular bodies (MVB) and late endosomes had a higher labeling density than other organelles; however, evidence of complete transcytosis, beyond the outer plasma membrane of endothelial cells, was found in all sections. Accumulation of TNF α in MVB in cells beyond the basal lamina (Fig.5B) is suggestive of intact or largely intact transcytosed TNF α , since free iodine does not accumulate in tissue and organelles of the endocytotic pathway. At this time point (30 min), no localization of silver grains was observed in the Golgi complex. Taken together, these results show that a significant fraction of radiolabeled TNF α can cross the BBB within 30 min of iv injection in vivo.

4. DISCUSSION

Studies of the mechanism of TNF α trafficking are dependent on the sensitivity and specificity of the tags. In this study, we used ^{125}I and biotin as tracer molecules conjugated to TNF α . The smaller tags have advantages over colloid-gold, being less likely to alter biological activity and trafficking pathways, or to lead to nonspecific degradation of the labeled protein after clathrin-mediated endocytosis. The mature protein of TNF α is a 17 kD protein with a total of 8 tyrosine and 3 histidine residues. Even if di-iodination occurred on each residue, the addition of iodine would increase the molecular mass less than 15 %. Similarly, biotinylation occurs at the lysine residues; poly-biotinylation of TNF α might increase the size by only 2684 Da (less than 14 % of the molecular weight) and is much less likely to change the conformation than colloidal gold, which has a diameter of 9 – 14 nm compared with the 2 nm TNF α protein itself. The probability of small tags affecting critical amino acid residues that influence receptor binding is low, involving only a small fraction of iodinated molecules, allowing adequate tracing (von Bartheld 2001).

The use of tagged molecules also overcomes the problem of the potential induction of endogenous TNF α that would have interfered with interpretations based on immunostaining with an anti-TNF α antibody. Furthermore, there is signal amplification of biotinylated TNF α by streptavidin-Qdot which confers higher stability with more resistance to photobleaching (Lidke et al. 2004; Michalet et al. 2005). With these improved techniques, we set out to determine what distinguishes the endocytosed TNF α that is destined to avoid degradation and instead completes its passage across the BBB cell.

RBE4 cells of rat brain origin, immortalized by an adenovirus, retain sufficient features of the BBB to be widely used as a model for in-vitro studies of the BBB (Durieu-Trautmann et al. 1994; Rist et al. 1997; Etienne-Manneville et al. 2000; Pan et al. 2005). Since TNF α crosses the BBB in mice by receptor-mediated transport (Pan and Kastin 2002), we first determined the presence of TNFR1 and TNFR2 and showed that the cells provided an adequate model to study TNF α trafficking. Binding and internalization of ^{125}I -TNF α was at least 4-times higher than the nonspecific background seen in the presence of excess unlabeled TNF α at 4°C. Endocytosis was inversely correlated with binding and showed rapid influx kinetics, with a half-life of about 4 min.

Caveolae are present at both the cell surface and in the cytoplasm, particularly in the Golgi complex (Conrad et al. 1995; Tagawa et al. 2005). Our finding of rapid, ligand-induced colocalization of TNFR1 and TNFR2 with caveolin-1 suggests the involvement of caveolae in the receptor-mediated endocytosis of TNF α . It has been shown that TNF α receptor-associated factor 2 (TRAF2) interacts with caveolin-1 in other cellular models (Feng et al. 2001; Cao et al. 2002) and probably is involved in the recruitment of caveolae upon TNF α binding. The results are consistent with reports in non-BBB cells showing that TNFR1 endocytosis can be mediated by either caveolae (Bradley et al. 1993; D'Alessio et al. 2005) or clathrin-coated pits (Mosselmans et al. 1988). Although earlier studies showed a predominant role of caveolae in transcytosis, as occurs in the blood-lung barrier (Milici et al. 1987; Ghitescu and Bendayan 1992), both mechanisms may lead to transcytosis (Abulrob et al. 2005).

Exocytosis of intact ^{125}I -TNF α was shown by kinetic assays and HPLC analysis. It was supported by the imaging evidence that biotinylated TNF α did not follow the degradative pathway through lysosomes and proteasomes after 20 min of continuous internalization. In RBE4 cells loaded with ^{125}I -TNF α after 20 min of endocytosis, about 0.2% of the internalized TNF α could be recovered in the exocytosis medium in intact form. We have shown that the transporting receptors for TNF α are mainly present at the apical surface (Gutierrez et al. 1993; Pan et al. 1997b, 2003a; Pan and Kastin 2002). However, the mechanisms leading to exocytosis in endothelial cells remain largely unexplored.

Our radiotracer studies used 2 – 5 pg/ml of TNF α , in contrast to the much larger circulating TNF α concentration of about 5 ng/ml in normal mice and 88 ng/ml in double receptor knockout mice, both 2 h after a single dose of LPS (400 μg iv) (Peschon et al. 1998). High doses of TNF α have many effects on endothelial cells and intercellular junctions, including alterations in cytoskeletal organization, tight junction protein expression, and production of the serine proteases involved in BBB disruption, tissue remodeling, and neural plasticity (Stolphen et al. 1986; Dobbie et al. 1999; Rosenberg 2002). TNF α also induces cerebral endothelial cell expression of genes involved in such processes as cell adhesion, chemotaxis, apoptosis, transcriptional regulation, and neuroprotection (Franzén et al. 2003).

The specific transport of TNF α itself, however, reveals another layer of biological significance. Our studies in mice have shown that the transported TNF α does not cause acute BBB disruption, as determined by co-administered vascular markers. The physiological significance of the selective receptor-mediated transport of TNF α is clearly demonstrated in animal models including spinal cord injury, brain trauma, and stroke (Pan et al. 1999, 2003b, 2003c, 2006; Pan and Kastin 2001, 2002). At specific times and in particular CNS regions, upregulation of the TNF α transport system has been demonstrated by functional transport assays, and the substrate of such regulation shown by increased TNF α receptor mRNA and protein expression. As TNF α plays a pivotal role in neuroregeneration and its permeation across the BBB can be specifically regulated, its receptor-mediated transport offers a novel target for therapeutic intervention. Such implications add relevance to our current study of TNF α trafficking in cerebral vascular endothelial cells.

With quantitative EM autoradiography, we now show that ^{125}I -TNF α can cross the BBB to arrive at adjacent cells within the brain parenchyma. The fraction (36%) of TNF α that transcytosed the BBB based on quantitative EM autoradiographic tracing was higher than the fraction exocytosed by RBE4 cells (11%), indicating that measurements in-vitro may underestimate the amounts that transcytose in-vivo. The parts of our study involving EM autoradiography do not prove that the accumulating radiolabeled protein was intact, although free iodine is not retained during tissue processing. Whether the TNF ligand remained intact, therefore, was addressed by our HPLC experiments which showed that most of the TNF α was not degraded.

In summary, TNF α can transcytose endothelial cells in-vivo and shows vesicular trafficking in RBE4 cells involving membrane microdomains. A significant amount (about 11%) of TNF α can exit even partially depolarized cerebral microvessel cells in intact form, and a greater fraction (36%) can reach brain parenchyma in mice 30 min after iv injection. The potential effect of peripheral TNF α on CNS function, therefore, is vast.

Acknowledgements

RBE4 cells were originated in the laboratory of Dr. Pierre-Olivier Couraud (Institute of Cochin, Paris, France). We thank Dr. Hong Tu for stimulating discussions, Ms. Yongmei Yu for technical assistance, and Ms. Loula Burton for excellent editorial support.

Reference List

- Abulrob A, Sprong H, Van Bergen en Henegouwen P, Stanimirovic D. The blood-brain barrier transigrating single domain antibody: mechanisms of transport and antigenic epitopes in human brain endothelial cells. *J Neurochem* 2005;95:1201–1214. [PubMed: 16271053]
- Banks WA, Farr SA, La Scola ME, Morley JE. Intravenous human interleukin-1 α impairs memory processing in mice: dependence on blood-brain barrier transport into posterior division of the septum. *J Pharmacol Exp Ther* 2001;299:536–541. [PubMed: 11602664]
- Banks WA, Ortiz L, Plotkin SR, Kastin AJ. Human interleukin (IL)1 α , murine IL-1 α and murine IL-1 β are transported from blood to brain in the mouse by a shared saturable mechanism. *J Pharmacol Exp Ther* 1991;259:988–996. [PubMed: 1762091]
- Bradley JR, Johnson DR, Pober JS. Four different classes of inhibitor of receptor-mediated endocytosis decrease tumor necrosis factor-induced gene expression in human endothelial cells. *J Immunol* 1993;150:5555.
- Cao H, Courchesne WE, Mastick CC. A phosphotyrosine-dependent protein interaction screen reveals a role for phosphorylation of caveolin-1 on tyrosine 14: recruitment of C-terminal Src kinase. *J Biol Chem* 2002;277:8771–8774. [PubMed: 11805080]
- Conrad PA, Smart EJ, Ying YS, Anderson RGW, Bloom GS. Caveolin Cycles Between Plasma-Membrane Caveolae and the Golgi-Complex by Microtubule-Dependent and Microtubule-Independent Steps. *Journal of Cell Biology* 1995;131:1421–1433. [PubMed: 8522601]
- D'Alessio A, Al Lamki RS, Bradley JR, Pober JS. Caveolae participate in tumor necrosis factor receptor 1 signaling and internalization in a human endothelial cell line. *Am J Pathol* 2005;166:1273–1282. [PubMed: 15793306]
- Ding AH, Sanchez E, Srimal S, Nathan CF. Macrophage rapidly internalize their tumor necrosis factor receptors in response to bacterial lipopolysaccharide. *J Biol Chem* 1989;264:3924–3929. [PubMed: 2537297]
- Dobbie MS, Hurst RD, Klein NJ, Surtees RA. Upregulation of intercellular adhesion molecule-1 expression on human endothelial cells by tumour necrosis factor- α in an in vitro model of the blood-brain barrier. *Brain Res* 1999;830:330–336. [PubMed: 10366690]
- Durieu-Trautmann O, Chaverot N, Cazaubon S, Strosberg D, Couraud PO. Intercellular adhesion molecule 1 activation induces tyrosine phosphorylation of the cytoskeleton-associated protein cortactin in brain microvessel endothelial cells. *J Biol Chem* 1994;269:12536–12540. [PubMed: 7909803]
- Etienne-Manneville S, Manneville JB, Adamson P, Wilbourn B, Greenwood J, Couraud PO. ICAM-1-coupled cytoskeletal rearrangements and transendothelial lymphocyte migration involve intracellular calcium signaling in brain endothelial cell lines. *J Immunol* 2000;165:3375–3383. [PubMed: 10975856]
- Feng X, Gaeta ML, Madge LA, Yang JH, Bradley JR, Pober JS. Caveolin-1 associates with TRAF2 to form a complex that is recruited to tumor necrosis factor receptors. *J Biol Chem* 2001;276:8341–8349. [PubMed: 11112773]
- Franzén B, Duvefelt K, Jonsson C, Engelhardt B, Ottervald J, Wickman M, Yang Y, Schuppe-Koistinen I. Gene and protein expression profiling of human cerebral endothelial cells activated with tumor necrosis factor- α . *Mol Brain Res* 2003;115:130–146. [PubMed: 12877984]

- Ghitescu L, Bendayan M. Transendothelial transport of serum albumin: a quantitative immunocytochemical study. *J Cell Biol* 1992;117:745–755. [PubMed: 1577854]
- 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. [PubMed: 8370768]
- Higuchi M, Aggarwal BB. TNF induces internalization of the p60 receptor and shedding of the p80 receptor. *J Immunol* 1994;152:3550–3558. [PubMed: 8144934]
- Johanson, CE. Ventricles and cerebrospinal fluid. In: Conn, PM., editor. *Neuroscience in Medicine*. JBLippincott; Philadelphia : 1995. p. 171-196.
- Jones SJ, Ledgerwood EC, Prins JB, Galbraith J, Johnson DR, Pober JS, Bradley JR. TNF recruits TRADD to the plasma membrane but not the *trans*-Golgi network, the principal subcellular location of TNF-R1. *J Immunol* 1999;162:1042–1048. [PubMed: 9916731]
- Ledgerwood EC, Prins JB, Bright NA, Johnson DR, Wolfreys K, Pober JS, O’Rahilly S, Bradley JR. Tumor necrosis factor is delivered to mitochondria where a tumor necrosis factor-binding protein is localized. *Lab Invest* 1998;78:1583–1589. [PubMed: 9881958]
- Lidke DS, Nagy P, Heintzmann R, Arndt-Jovin DJ, Post JN, Grecco HE, Jares-Erijman EA, Jovin TM. Quantum dot ligands provide new insights into erbB/HER receptor-mediated signal transduction. *Nature Biotechnology* 2004;22:198–203.
- McGraw, TE.; Subtil, A. *Current Protocols in Cell Biology*. John Wiley & Sons, Inc; 1999. Protein trafficking; p. 15.3.1-15.3.23.
- Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 2005;307:538–544. [PubMed: 15681376]
- Milici AJ, Watrous NE, Stukenbrok H, Palade GE. Transcytosis of albumin in capillary endothelium. *J Cell Biol* 1987;105:2603–2612. [PubMed: 3320050]
- Mosselmans R, Hepburn A, Dumont JE, Fiers W, Galand P. Endocytic pathway of recombinant murine tumor necrosis factor in L-929 cells. *J Immunol* 1988;141:3096–3100. [PubMed: 2902145]
- Pan W, Banks WA, Kastin AJ. BBB permeability to ebitride and TNF in acute spinal cord injury. *Exp Neurol* 1997a;146:367–373. [PubMed: 9270046]
- Pan W, Banks WA, Kastin AJ. Permeability of the blood-brain and blood-spinal cord barriers to interferons. *J Neuroimmunol* 1997b;76:105–111. [PubMed: 9184639]
- Pan W, Banks WA, Kennedy MK, Gutierrez EG, Kastin AJ. Differential permeability of the BBB in acute EAE: enhanced transport of TNF- α . *Am J Physiol* 1996;271:E636–E642. [PubMed: 8897850]
- Pan W, Csernus B, Kastin AJ. Upregulation of p55 and p75 receptors mediating TNF alpha transport across the injured blood-spinal cord barrier. *J Mol Neurosci* 2003a;21:173–184. [PubMed: 14593216]
- Pan W, Ding Y, Yu Y, Ohtaki H, Nakamachi T, Kastin AJ. Stroke upregulates TNF alpha transport across the blood-brain barrier. *Exp Neurol* 2006;198:222–233. [PubMed: 16412421]
- Pan W, Kastin AJ. Upregulation of the transport system for TNF α at the blood-brain barrier. *Arch Physiol Biochem* 2001;109:350–353. [PubMed: 11935370]
- Pan W, Kastin AJ. TNF α transport across the blood-brain barrier is abolished in receptor knockout mice. *Exp Neurol* 2002;174:193–200. [PubMed: 11922661]
- Pan W, Kastin AJ, Bell RL, Olson RD. Upregulation of tumor necrosis factor α transport across the blood-brain barrier after acute compressive spinal cord injury. *J Neurosci* 1999;19:3649–3655. [PubMed: 10212323]
- Pan W, Kastin AJ, McLay RN, Rigai T, Pick CG. Increased hippocampal uptake of TNF α and behavioral changes in mice. *Exp Brain Res* 2003b;149:195–199. [PubMed: 12610687]
- Pan W, Yu Y, Cain CM, Nyberg F, Couraud PO, Kastin AJ. Permeation of growth hormone across the blood-brain barrier. *Endocrinol* 2005;146:4898–4904.
- Pan W, Zadina JE, Harlan RE, Weber JT, Banks WA, Kastin AJ. Tumor necrosis factor α : a neuromodulator in the CNS. *Neurosci Biobehav Rev* 1997c;21:603–613. [PubMed: 9353794]
- Pan W, Zhang L, Liao J, Csernus B, Kastin AJ. Selective increase in TNF α permeation across the blood-spinal cord barrier after SCI. *J Neuroimmunol* 2003c;134:111–117. [PubMed: 12507778]

- Peschon JJ, Torrance DS, Stocking KL, Glaccum MB, Otten C, Willis CR, Charrier K, Morrissey PJ, Ware CB, Mohler KM. TNF receptor-deficient mice reveal divergent roles for p55 and p75 in several models of inflammation. *J Immunol* 1998;160:943–952. [PubMed: 9551933]
- Plotkin SR, Banks WA, Kastin AJ. Comparison of saturable transport and extracellular pathways in the passage of interleukin- 1α across the blood-brain barrier. *J Neuroimmunol* 1996;67:41–47. [PubMed: 8707929]
- Rist RJ, Romero IA, Chan MWK, Couraud PO, Roux R, Abbott NJ. F-actin cytoskeleton and sucrose permeability of immortalised rat brain microvascular endothelial cell monolayers: effect of cyclic AMP and astrocytic factors. *Brain Res* 1997;768:10–18. [PubMed: 9369295]
- Rosenberg GA. Matrix metalloproteinases in neuroinflammation. *Glia* 2002;39:279–291. [PubMed: 12203394]
- Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschrander M, Adam D, Mentlein R, Kabelitz D, Schütze S. Compartmentalization of TNF receptor 1 signaling: Internalized TNF receptosomes as death signaling vesicles. *Immunity* 2004;21:415–428. [PubMed: 15357952]
- Schnitzer JE. Caveolae: from basic trafficking mechanisms to targeting transcytosis for tissue-specific drug and gene delivery in vivo. *Adv Drug Deliv Rev* 2001;49:265–280. [PubMed: 11551399]
- Schütze S, Machleid T, Adam D, Schwandner R, Wiegmann K, Kruse ML, Heinrich M, Wickel M, Krönke M. Inhibition of receptor internalization by monodansylcadaverine selectively blocks p55 tumor necrosis factor receptor death domain signaling. *J Biol Chem* 1999;274:10203–10212. [PubMed: 10187805]
- Shurety W, Pagan JK, Prins JB, Stow JL. Endocytosis of uncleaved tumor necrosis factor- α in macrophages. *Lab Invest* 2001;81:107–117. [PubMed: 11204269]
- Stolphen AH, Guinan EC, Fiers W, Pober JS. Recombinant tumor necrosis factor and immune interferon act singly and in combination to reorganize human vascular endothelial cell monolayers. *Am J Pathol* 1986;123:16–24. [PubMed: 2421578]
- Tagawa A, Mezzacasa A, Hayer A, Longatti A, Pelkmans L, Helenius A. Assembly and trafficking of caveolar domains in the cell: caveolae as stable, cargo-triggered, vesicular transporters. *Journal of Cell Biology* 2005;170:769–779. [PubMed: 16129785]
- von Bartheld, CS. Tracing with radiolabeled neurotrophins. In: Rush, RA., editor. *Methods in Molecular Biology*. Humana Press ; Totowa NJ : 2001. p. 195-216.
- von Bartheld CS. Axonal transport and neuronal transcytosis of trophic factors, tracers and pathogens. *J Neurobiol* 2004;58:295–314. [PubMed: 14704960]
- Vuk-Pavlovic S, Kovach JS. Recycling of tumor necrosis factor- α receptor in MCF-7 cells. *FASEB J* 1989;3:2633–2640. [PubMed: 2556313]

Fig1. Biochemical assays of $^{125}\text{I-TNF}\alpha$ trafficking in RBE4 cells

A. After 2 h binding at 4 °C, surface binding and endocytosis of surface-bound $^{125}\text{I-TNF}\alpha$ was determined 0 - 90 min after rapid warming of RBE4 cells to 37 °C. In this pulse-chase experiment, a significant ($p < 0.005$) inverse correlation was seen between the increase in endocytosis and decrease in surface binding of $^{125}\text{I-TNF}\alpha$. The half-time dissociation of the ligand from the cell surface was 4.38 ± 0.02 min.

B. After continuous uptake of $^{125}\text{I-TNF}\alpha$ at 37 °C for 0, 5, 10, 20, or 60 min, the amount of radioactivity remaining at the surface of RBE4 cells and that endocytosed was determined. Maximal potential internalization is the sum of the endocytosed $^{125}\text{I-TNF}\alpha$ together with that remaining at the cell surface, and shows a significant increase at 60 min. *: $p < 0.05$; ***: $p < 0.005$ compared with the 0 time group (binding without specific endocytosis).

C. Saturability of the binding and endocytosis of $^{125}\text{I-TNF}\alpha$ after inhibition in the presence of 200-fold excess unlabeled $\text{TNF}\alpha$: Maximal potential internalization reflects both pools of $^{125}\text{I-TNF}\alpha$. ***: $p < 0.005$ when compared with the $^{125}\text{I-TNF}\alpha$ only control group examined at the same time point. +++: $p < 0.005$ when compared with the 0 time group studied at 4 °C instead of 37 °C.

Fig2. Confocal images of subcellular localization of TNFR1 and TNFR2 in RBE4 cells
 A. Distribution of receptors in the resting state showed that TNFR1 (red) was partially colocalized with the Golgi complex marker βCOP (green) whereas R2 was not.
 B. Partial colocalization of TNFR1 and TNFR2 (red) with caveolin-1 (green) in RBE4 cells after 2 min of TNFα treatment (5 ng/ml). Colocalization was not seen in untreated cells.

Fig3. Endocytosis of biotinylated TNFα at different time points after endocytosis shown by streptavidin-Qdot

A. Vesicular pattern of distribution of the signal at 2, 10, and 20 min after endocytosis, seen only with biotinylated TNFα, but not in the negative controls with only biotin or without incubation with streptavidin-Qdot.

B. Lack of colocalization of biotinylated TNFα with lamp-1 (lysosome marker) or the 20 s protein (proteasome marker) 20 min after endocytosis.

Fig4. Exocytosis of ^{125}I -TNF α by RBE4 cells after continuous endocytosis for 20 min

A. Release of ^{125}I -TNF α to the medium at 2, 5, 10, 20, 30, and 60 min after initiation of exocytosis. Significantly more radioactivity was released into the transport buffer at later times (30 and 60 min), and significant degradation was seen only at 60 min (inset). ***: $p < 0.005$ compared with the 0 time control.

B. HPLC showed that more than 50% of the radioactivity recovered 30 min after initiation of exocytosis represented intact ^{125}I -TNF α . The bar indicates the elution position of the stock solution.

Fig5.

EM autoradiography of ultrathin sections through mouse cerebral cortex blood vessels 30 min after iv injection of ^{125}I -TNF α showing two representative images (A, B). Silver grains accumulated primarily over endothelial cells (E1), and occasionally over presumptive smooth muscle cells (M1) and pericytes (P1). The brain parenchyma contains a significant fraction of silver grains (asterisks), outside the basal lamina (indicated by small arrows), showing complete passage of TNF α across the BBB by transcytosis. For quantification, see Table 1. The silver grains representing radiolabeled ^{125}I -TNF α in endothelial cells and presumptive pericytes were associated with coated (CV) and non-coated vesicles (V), early endosomes (EE), and endoplasmic reticulum (ER) in these representative images. Some silver grains in the brain parenchyma were associated with multivesicular bodies (MVB, panel B). Scale bar = 1 μm .

Table 1
Distribution of radiolabeled TNF α in cerebral cortex 30 min after iv injection*

Structure	Silver grains	[%]
Vessel lumen	79	24.7
Inner membrane of endothelial cell	23	7.2
Endothelial cell (interior)	76	23.7
Outer membrane of endothelial cell	15	4.7
Pericyte (presumptive)	5	1.6
Smooth muscle cell (presumptive)	5	1.6
Brain parenchyma within 15 μ m from vessel wall	117	36.6

* Total = 320 silver grains from 127 images in 3 tissue blocks from 2 mice. Silver grains were categorized based on the location of the center of each silver grain.