

Both TNF receptors are required for direct TNF-mediated cytotoxicity in microvascular endothelial cells

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The conditions under which tumor necrosis factor- α (TNF) induces apoptosis in primary microvascular endothelial cells (MVEC) were investigated. In the absence of sensitizing agents, TNF induced apoptosis after 3 days of incubation in confluent MVEC. In contrast, upon addition of the transcriptional inhibitor actinomycin D (Act. D), confluence was no longer required and apoptosis occurred already after 16 h. To assess the role of either TNF receptor (TNFR) type in apoptosis, MVEC isolated from mice genetically deficient in TNFR1 (*Tnfr1*[°] mice) or TNFR2 (*Tnfr2*[°] mice) were incubated with TNF in the presence or absence of Act. D. Under sensitized conditions, *Tnfr2*[°] MVEC were lysed like controls, whereas *Tnfr1*[°] MVEC were completely resistant, indicating an exclusive role for TNFR1. In contrast, in the absence of Act. D, confluent monolayers of wild-type cells were lysed by TNF, but both *Tnfr1*[°] and *Tnfr2*[°] MVEC were resistant to TNF-mediated toxicity, indicating a requirement for both TNFR types. Overexpression of the anti-apoptotic protein bcl-xL in MVEC led to a protection against the direct, but not the sensitized cytotoxicity of TNF. In conclusion, in pathophysiologically relevant conditions, both TNFR appear to be required for TNF-induced apoptosis in MVEC.

Key words: TNF / Endothelial cell / Apoptosis / bcl-xL

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1 Introduction

The capacity of TNF to induce hemorrhagic necrosis of certain tumors *in vivo* [1] has been shown to be partly mediated by endothelial injury of the tumor microvasculature [2, 3]. Thus, microvascular endothelial cells (MVEC) are important target cells for the tumor necrotic activity of TNF. Moreover, MVEC are critical in the devel-

opment of tissue lesions associated with an overproduction of TNF, as can be found in septic shock (lung MVEC) [4] or cerebral malaria (brain MVEC) [5]. *In vivo* observations have revealed that MVEC are more sensitive to TNF than large vessel endothelial cells (LVEC) following systemic infusion [6]. On the other hand, *in vitro*, lung MVEC have been reported to be resistant towards the direct lytic activity of TNF [7], in contrast to LVEC, which various groups found to undergo apoptosis in response to TNF [7–10].

To investigate whether TNF can lyse MVEC under specific conditions, we isolated brain MVEC and tested them for their sensitivity to TNF, using confluent versus sub-confluent conditions. Moreover, we wished to

[1 18599]

Abbreviations: MVEC: Microvascular endothelial cells
LVEC: Large vessel endothelial cells Act. D: Actinomycin D
CHX: Cycloheximide ECGS: Endothelial cell growth supplement
m: Mouse h: Human wt: Wild-type

assess the role of both TNFR in TNF-induced cytotoxicity. Since MVEC express both the 55-kDa (TNFR1) and the 75-kDa (TNFR2) TNFR, we assessed the importance of each of these receptors by using MVEC isolated from TNFR gene-deficient mice [11, 12]. In this respect, it should be noted that TNFR1 contains a death domain [13, 14], whereas TNFR2 does not. However, the latter TNFR was recently shown to be implicated in apoptosis induction in activated T cells, and this occurred through inhibition of the expression of the anti-apoptotic protein bcl-xL [15].

2 Results

2.1 Either confluence or transcriptional inhibition are required for cytolytic activity of TNF on MVEC

Under sub-confluent conditions (3×10^4 cells/well at the start of the assay), no lysis of CBA/J brain MVEC was detectable after 24, 48, 72 or 96 h of incubation with mouse (m)TNF (up to 1000 IU/ml), as measured by ethidium homodimer incorporation (data for 72 h incubation are shown in Fig. 1A). In contrast, under initial confluent conditions (5×10^4 cells/well), these cells were lysed after 72 h of incubation with mTNF in a dose-dependent manner (Fig. 1A). In the presence of the transcriptional inhibitor actinomycin D (Act. D) ($1 \mu\text{g/ml}$) however, brain MVEC became sensitive to TNF-induced cytolysis, even under sub-confluent conditions, whereas Act. D alone had no effect (Fig. 1B). This cytolysis was already detectable after 24 h of incubation (Fig. 1B). When the protein synthesis inhibitor cycloheximide (CHX; $3 \mu\text{g/ml}$) was added 4 h prior to TNF and Act. D, *i.e.* under conditions that are optimal for protein synthesis inhibition [16], the level of TNF and Act. D-induced MVEC cytolysis was reduced to that observed with CHX alone, *i.e.* $\sim 15\%$ (Fig. 1B). All these results were confirmed using murine CBA/J lung MVEC and bovine adrenocortical MVEC (data not shown).

2.2 TNF kills MVEC by means of apoptosis

Apoptosis was assessed by means of fluorescence microscopy by counting cells stained with acridine orange which displayed the typical characteristics of apoptosis, such as chromatin condensation (seen as intensified fluorescence), contraction of the cytoplasm and the formation of projections or blebs. Apoptosis was induced in confluent brain MVEC treated for 48 h with 10 ng/ml mTNF. Under these conditions, $37 \pm 5\%$ of the TNF-treated cells were apoptotic, compared to $5 \pm 2\%$ in the control group (Fig. 2). In the presence of Act. D,

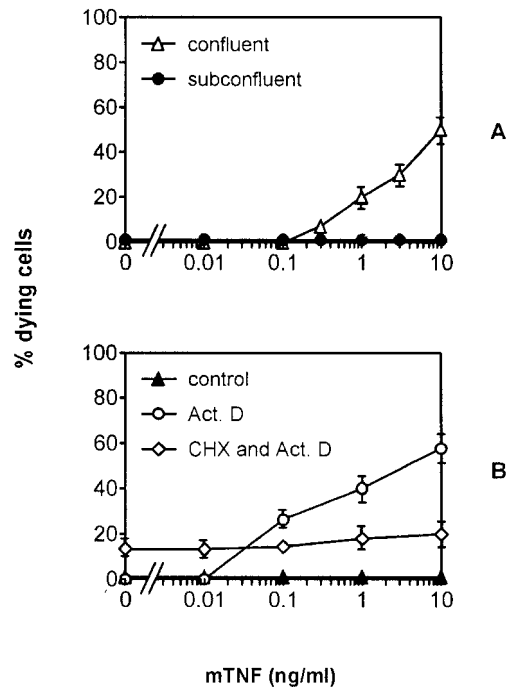


Figure 1. Conditions under which TNF induces toxicity in MVEC. (A) Cytotoxic activity of mTNF on CBA/J brain MVEC under confluent (5×10^4 cells/well) vs. sub-confluent (3×10^4 cells/well) conditions after 72 h of incubation. (B) Cytolytic activity of mTNF on CBA/J brain MVEC in the presence or absence of $1 \mu\text{g/ml}$ Act. D and/or $3 \mu\text{g/ml}$ CHX after 24 h of incubation. Cells were pretreated for 15 min with Act. D and for 4 h with CHX before the addition of mTNF. All experiments represent the means of triplicates of three experiments \pm SD.

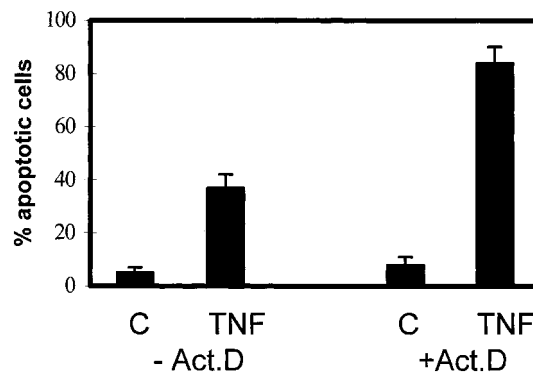


Figure 2. mTNF (10 ng/ml) induces apoptosis in brain MVEC. The number of apoptotic cells was assessed by acridine orange staining after 48 h of incubation with 10 ng/ml mTNF or after 16 h of incubation with 10 ng/ml mTNF and $1 \mu\text{g/ml}$ Act. D. The data represent the means of quadruplicates \pm SD.

apoptosis could be detected from 6 h on ($20 \pm 3\%$ in the TNF-treated group vs. $4 \pm 1\%$ in the Act. D controls), and after 16 h, $84 \pm 6\%$ of cells were affected in the mTNF-treated group vs. $8 \pm 3\%$ in the control group (Fig. 2).

2.3 Comparison of cytotoxicity of human versus mTNF in mouse brain MVEC

As shown in Fig. 3A, human (h)TNF, which does not interact with mouse TNFR2 [17], had a 10-fold reduced capacity in the presence of Act. D to induce apoptosis in CBA/J brain MVEC, which express both TNFR (data not shown). In sharp contrast, in the absence of Act. D, hTNF had a more than 100-fold reduced capacity, as compared to mTNF, to induce cell killing (Fig. 3B). These results thus suggested that TNFR2 is required for TNF-induced cytotoxicity in the absence, but not in the presence of Act. D. To further substantiate this hypothesis, we studied TNF-induced cytotoxicity in MVEC isolated from *Tnfr1*[°] and *Tnfr2*[°] mice.

2.4 TNFR expression in brain MVEC isolated from wt, *Tnfr1*[°] and *Tnfr2*[°] mice

Brain MVEC isolated from B6 × 129 wild-type (wt) mice expressed both types of TNFR, as assessed by a cell-based immunofluorescence assay. Cells isolated from *Tnfr1*[°] mice and *Tnfr2*[°] mice expressed the remaining receptor type at a level comparable to wt MVEC (Fig. 4). The difference in sensitivity to TNF between confluent and sub-confluent cells was not attributable to a different level of expression of either TNFR: we did not detect any increased level of either receptor under confluent vs. sub-confluent conditions (data not shown).

2.5 Role of TNFR in sensitized versus direct TNF cytotoxicity

When treated with both mTNF and Act. D in a 24-h assay, brain MVEC from *Tnfr1*[°] mice were completely resistant to cytolysis, whereas cells isolated from *Tnfr2*[°] mice behaved in a manner comparable to that of cells isolated from control mice (Fig. 5A), which is consistent with results presented in Fig. 3A. Act. D alone had no toxic effect on the MVEC from either wt, *Tnfr1*[°] or *Tnfr2*[°] mice. In contrast to the experiments performed in the presence of Act. D, brain MVEC from both *Tnfr1*[°] and *Tnfr2*[°] mice, incubated with mTNF for 72 h, were protected against cytolysis, whereas wt cells were lysed (Fig. 5B). These results were confirmed using CBA/J lung MVEC (data not shown).

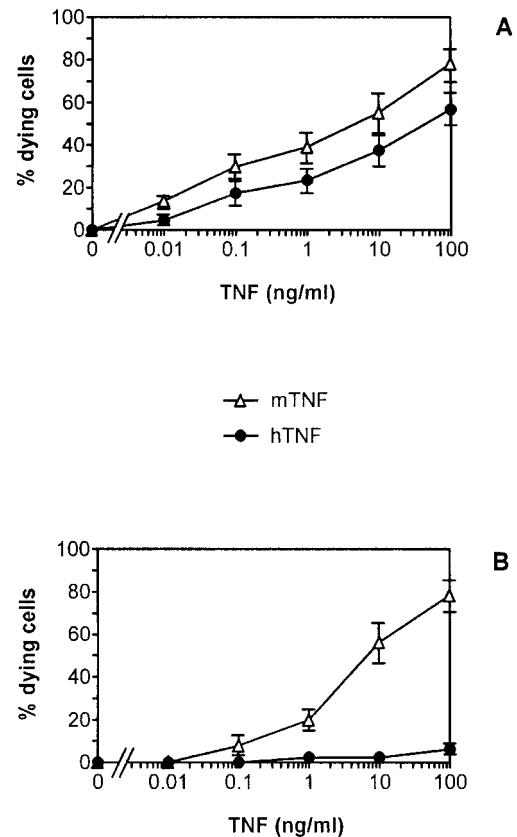


Figure 3. Comparison of cytotoxic activity of hTNF vs. mTNF in CBA/J brain MVEC in the presence (A) or absence (B) of Act. D. All values represent the means of quadruplicates of two experiments \pm SD.

2.6 Overexpression of bcl-xL protects against direct, but not against sensitized TNF cytotoxicity in MVEC

Recently, TNFR2 was shown to be implicated in apoptosis of activated T cells by means of inhibiting the expression of the anti-apoptotic protein bcl-xL [15]. Our previous results had indicated that TNFR2 is implicated in the direct, but not in the sensitized cytotoxicity of TNF in MVEC. Therefore, we wanted to investigate whether overexpression of bcl-xL could protect against TNF cytotoxicity implicating TNFR2. Transfected CBA brain MVEC showed a marked increase in expression of bcl-xL (Fig. 6B), as compared to non-transfected controls (Fig. 6A). The transfected cells no longer required the presence of endothelial cell growth supplement (ECGS) to proliferate and still efficiently responded to TNF-induced E-selectin expression (data not shown; Lucas et al., manuscript in preparation). After 24 h of incubation in the presence of Act. D, transfected MVEC, cultured either with or without ECGS, were as sensitive to TNF

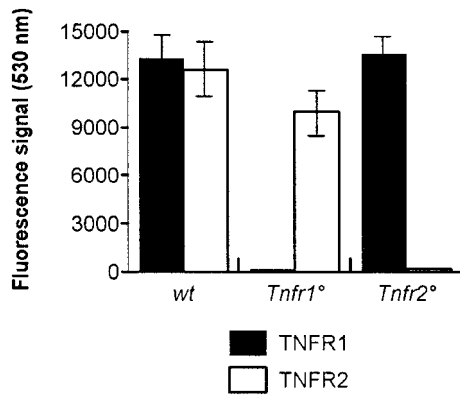


Figure 4. Expression levels of TNFR1 and TNFR2 in MVEC isolated from wt B6 × 129, *Tnfr1*[°] and *Tnfr2*[°] mice. The values represent the means of quadruplicates ± SD.

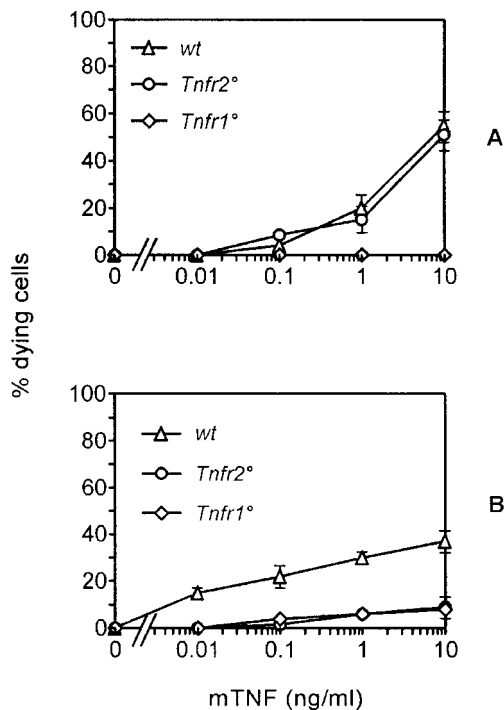


Figure 5. Comparison of sensitivity of brain MVEC isolated from wt B6 × 129 mice vs. *Tnfr1*[°] and *Tnfr2*[°] mice towards: (A) the combined lytic effect of mTNF and Act. D after 24 h of incubation (3×10^4 cells/well), and (B) the direct lytic effect of mTNF after 72 h of incubation (5×10^4 cells/well). All values represent the means of quadruplicates of three experiments ± SD.

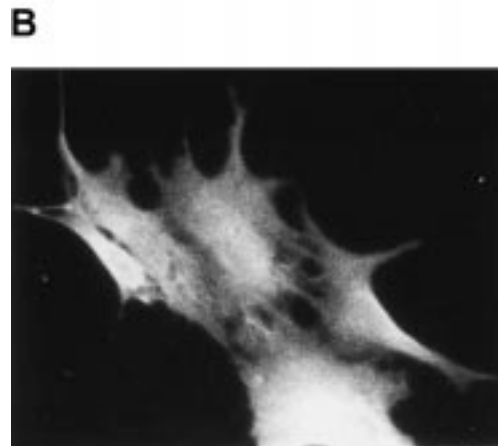
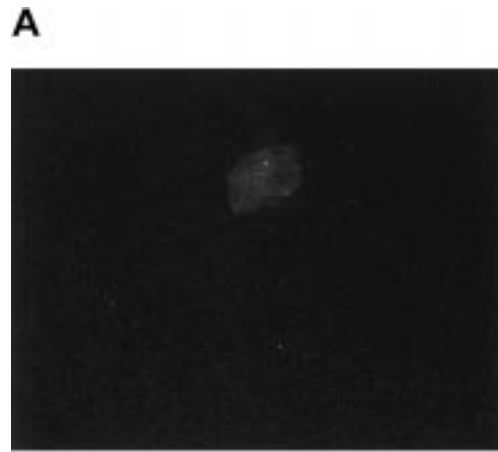


Figure 6. Expression levels of bcl-xL in (A) wt brain CBA/J MVEC and (B) pSFFV bcl-xL-transfected CBA/J brain MVEC.

cytotoxicity as the non-transfected cells (Fig. 7A). In sharp contrast, after 72 h of incubation, confluent monolayers of bcl-xL-transfected MVEC were resistant to direct TNF cytotoxicity, whereas the control cells or the cells microinjected with the control vector were efficiently lysed (Fig. 7B).

3 Discussion

In vivo, both TNFR appear to be involved in the systemic toxicity of TNF. Both *Tnfr1*[°] [11, 18, 19] and *Tnfr2*[°] mice [12] were shown to be resistant to lethal doses of mTNF, with resistance being more marked in the former [18]. Apart from being directly cytotoxic, TNF can indirectly cause tissue damage, e.g. by means of inducing adhesion molecules, such as ICAM-1, that increases the interaction between the endothelium and leukocytes [20, 21] or platelets [22]. Moreover, the decreased activation of

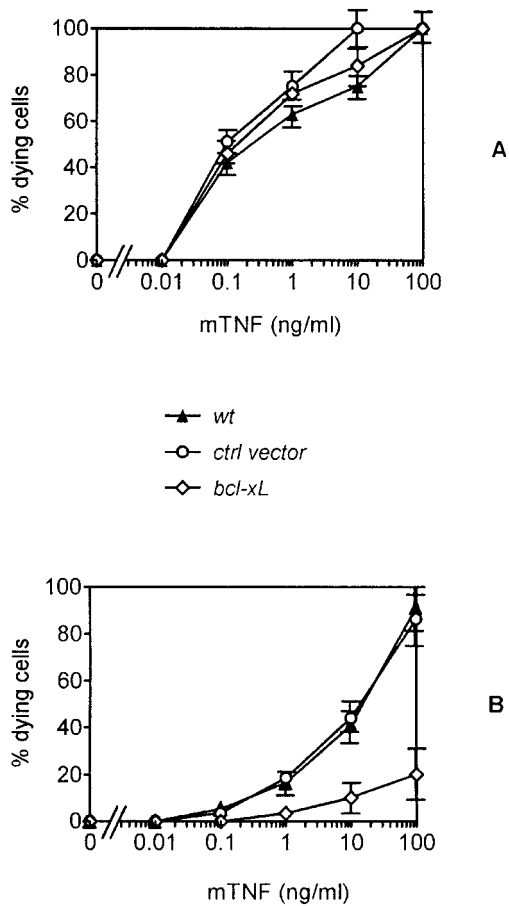


Figure 7. Comparison of sensitivity to (A) Act. D-sensitized and (B) direct mTNF cytotoxicity between wt, control vector-microinjected and bcl-xL-overexpressing CBA/J brain MVEC. All values represent the means of triplicates of three experiments \pm SD.

the integrin $\alpha v\beta 3$, an adhesion molecule which plays a key role in tumor angiogenesis, by the combined treatment of human endothelial cells with TNF and IFN- γ , was recently proposed to be one of the mechanisms implicated in the anti-vascular activity of these cytokines [23].

Here, we have addressed the direct *in vitro* toxicity of TNF on primary endothelial cells, which represent one of the major target cells relevant for TNF pathology *in vivo*. Most published studies have made use of LVEC that are both morphologically and functionally different from MVEC [24–26]. In this report, we have shown that mouse MVEC, which are more affected than LVEC during TNF infusion *in vivo* [6], can also be killed by mTNF *in vitro*, provided they are at confluence; following a 72-h incubation with mTNF under these conditions, the cells undergo apoptosis. However, under sub-confluent conditions, MVEC are not lysed directly by TNF. The latter

result is in accordance with earlier findings of experiments performed with bovine lung MVEC [7]. The reason for this confluence-dependent sensitivity of MVEC towards TNF remains unclear. We could not find significant changes in TNFR expression between confluent and sub-confluent cultures (data not shown), as was reported for epithelial and myeloid cells [27]. Neither could we find a role for membrane-bound TNF, which should only be active during cell-cell contact and was shown to be induced by exogenously added TNF in HeLa cells [28]. In this respect, we could not inhibit cytotoxicity induced by hTNF in murine MVEC with neutralizing antibodies towards membrane-bound mTNF (kind gift from M. Grell and K. Pfizenmaier, University of Stuttgart; data not shown), arguing against a role for endogenously produced membrane-bound mTNF in cytotoxicity in these cells.

Since the pretreatment of various cell types with Act. D can either induce or increase sensitivity to TNF, results obtained in the presence of transcriptional inhibitors do not necessarily reflect the *in vivo* effect of TNF on these cells. We found that in the presence of Act. D, TNFR1 was necessary and sufficient to mediate TNF-induced apoptosis in MVEC. This result is in agreement with the reported resistance of hepatocytes isolated from *Tnfr1*⁰ mice against cytotoxicity induced by the combination of TNF and Act. D [16]. However, in contrast to confluent MVEC, TNF had no direct cytotoxic effect on hepatocytes. Moreover, the combined effect of TNF and Act. D disappeared after several *in vitro* passages of hepatocytes [16]. These results differ from our observations with MVEC, which are directly killed by TNF at confluence and the sensitivity of which to TNF and Act. D does not decrease upon serial passaging (tested for up to four passages). The observation that MVEC isolated from *Tnfr2*⁰ mice are still efficiently lysed by a combined TNF and Act. D treatment also indicates that TNFR1, still expressed in these cells, is biologically active. The same holds true for TNFR2 in *Tnfr1*⁰ MVEC, which still mediates up-regulation of ICAM-1 induced by membrane-bound TNF [29].

In contrast to the results obtained under conditions of transcriptional inhibition, both TNFR1 and TNFR2 are required for direct TNF-induced MVEC apoptosis after 72 h. This result is in agreement with a previous report that showed that both TNFR types are necessary to induce DNA fragmentation in U-937 cells and cytotoxicity in PC 60 cells [30, 31]. Thus, depending on the presence or absence of transcriptional inhibitors, different pathways could be implicated in the TNF-induced killing of MVEC. Likewise, others have provided evidence that direct TNF-mediated cytotoxicity is genetically, pharmacologically, and temporally distinct from the cytotoxicity

mediated by TNF during protein synthesis inhibition [32]. Recently, TNF was shown to trigger apoptosis in CHX-treated human endothelial cells by means of an IL-1-converting enzyme (ICE)-like protease-dependent pathway, since this activity could be blocked by transfected CrmA protein or Z-VAD.fmk peptide, but not by transfected anti-apoptotic proteins such as bcl-2, bcl-xL or A1 [33]. In contrast, the co-treatment of these cells with TNF and C6-ceramide resulted in apoptosis inhibitable by transfected anti-apoptotic proteins but not by ICE-like protease inhibitors [33]. Since most studies assessing the cytotoxicity of TNF in endothelial cells have been performed in the presence of transcriptional or translational inhibitors, results from these experiments should be interpreted with caution, since a role for TNFR2 in cytotoxicity of endothelial cells would not be detected under these conditions.

Since TNFR2, in contrast to TNFR1, does not have a death domain [13, 14], its implication in apoptosis is rather surprising. However, a recent report showed that TNFR2 activation in activated T cells leads to a diminished expression of the anti-apoptotic protein bcl-xL in these cells [15]. Bcl-xL was recently shown to inhibit the association of the mammalian homologue of CED-4 (Apaf-1) and caspase 9, thus preventing the Apaf-1-mediated maturation of caspase-9 [34]. Our experiments with bcl-xL-overexpressing brain MVEC have indicated that these cells are still sensitive to TNF-mediated effects which are exclusively mediated by TNFR1, such as E-selectin induction (completely absent in MVEC from *Tnfr1*[°] mice) and Act. D combined cytotoxicity. The latter result is in contrast to recent findings that MVEC overexpressing the anti-apoptotic protein A1, of the bcl-2 protein family, are resistant to the combined mTNF and Act. D cytotoxicity [35]. Interestingly, bcl-xL-overexpressing MVEC were resistant to effects involving TNFR2, such as direct TNF cytotoxicity. Therefore, these results suggest the existence of a cross-talk between TNFR2 and bcl-xL in the absence of transcriptional inhibition: one factor, upon activation (TNFR2) or overexpression (bcl-xL), inhibits the effect of the other. Thus, the interaction between TNFR2 and bcl-xL might be one of the mechanisms by which this TNFR type is implicated in TNF cytotoxicity in MVEC. Although the basal expression levels of anti-apoptotic proteins such as bcl-2 and bcl-xL are consistently low or undetectable in endothelial cells, these levels can be significantly increased during viral infection of these cells. Indeed, the expression of bcl-2 has recently been shown to be activated in endothelial cells chronically expressing the human T cell lymphotropic virus type I, and this might be pathophysiologically relevant [36]. Alternatively, TNFR2 was proposed to increase the TNFR1-mediated apoptosis of HeLa cells by up-regulating cytosolic phospho-

lase A₂ levels [37]. Moreover, a recent study has shown that the absence of a TNFR2-associated protein TRAF2 can decrease the sensitivity of HeLa cells for TNF-induced killing by a factor of 1000. Since the TNFR2-associated signal transducer TRAF2 is a component of the TNFR1 signaling complex [38], these results indicate cross-talk between both TNFR for TNF-mediated cytotoxicity in these cells [28]. Since TNFR2, but not TNFR1 expression, is increased in lung MVEC in patients with acute respiratory distress syndrome [39], in hepatocytes from patients with chronic hepatitis B [40] and in brain MVEC from mice with cerebral malaria [20, 29], this indicates that this TNFR type, in addition to TNFR1, might also play an important role in TNF-mediated pathology.

In conclusion, our results show that proliferating MVEC are not killed by TNF alone, unless they are treated with transcriptional inhibitors. This combined TNF and Act. D toxicity is mediated by TNFR1. In contrast, in confluent monolayers of MVEC, TNF directly induces apoptosis, which is inhibited by either TNFR1 or TNFR2 deficiency. These results suggest that in physiologically relevant conditions, both TNFR are important for the direct cytotoxic effect of TNF on MVEC.

4 Materials and methods

4.1 Mice and cells

Male wt B6 × 129, as well as *Tnfr1*[°] [11] and *Tnfr2*[°] mice [12] of an age of 8–10 weeks were provided by F. Hoffmann-La Roche (Basel, Switzerland). Male CBA/J mice of the same age were purchased from Iffa Credo, Les Oncins, France.

Mouse brain MVEC were isolated as described [41], using magnetic beads (Dynabeads M-450, Dynal, Oslo, Norway), covalently bound to a purified rat-anti-mouse platelet-endothelial cell adhesion molecule (PECAM-1) mAb (kind gift from Dr. B. Imhof, Geneva). The cells were characterized for the presence of tight junctions, von Willebrand Factor expression, acetylated low density lipoprotein uptake and TNF-induced E-selectin expression (the latter being negative in cells lacking TNFR1). The cells were plated onto T25 flasks pre-coated with 2% gelatin (Sigma, Buchs, Switzerland) and cultured at 37 °C in a 5% CO₂ atmosphere. MVEC were passaged up to four times.

4.2 Buffers and reagents

MVEC were resuspended in DMEM containing 2 mM L-glutamine, 100 U/ml penicillin, 10 µg/ml streptomycin, 20% FCS, 40 U/ml heparin and 100 µg/ml ECGS (Sigma). Recombinant mTNF and hTNF (Innogenetics, Ghent, Belgium) had a specific activity of 2 × 10⁸ and 2 × 10⁷ IU/mg,

respectively. Act. D, CHX and acridine orange were obtained from Sigma, Buchs, Switzerland.

4.3 TNF cytotoxicity assay

An ethidium homodimer-1 incorporation assay was used to screen for lysis of endothelial cells. Ethidium homodimer-1 (Molecular Probes, Leiden, The Netherlands) is a high-affinity red fluorescent DNA dye that is only internalized through altered cell membranes. This assay has recently been shown to be more sensitive than the MTT incorporation assay when screening for TNF cytotoxicity [42] and has previously been used to screen for TNF-induced killing of MVEC [43]. Briefly, MVEC (3×10^5 cells/ml) kept in DMEM without phenol red (phenol red is a quencher of the ethidium homodimer signal) were incubated for 24 h in the presence of various concentrations of mTNF or hTNF and 1 μ g/ml Act. D. Alternatively, confluent monolayers of MVEC (5×10^5 cells/ml) were treated for 72 h with various concentrations of mTNF or hTNF. Subsequently, the MVEC were treated for 30 min at 37 °C with 8 mM ethidium homodimer solution. As controls, cells pretreated for 30 min at room temperature with 33 % ethanol (positive control) as well as cells incubated in medium (negative control) were included. The fluorescence signal was then read using 530 nm as excitation wavelength and 620 nm as emission wavelength in a CytoFluor® II fluorescence Multi-Well Plate Reader (PerSeptive Biosystems, MA), after which the percentage of dying cells was calculated, using the following formula: [(experimental signal) – (medium signal)]/[(ethanol signal) – (medium signal)] \times 100 %.

4.4 Apoptosis detection

Quantification of apoptotic cells was accomplished by acridine orange uptake as described [44, 45]. Cells were incubated with 1 μ g/ml acridine orange for 5 min and fixed in 4 % paraformaldehyde. Cellular morphology was subsequently assessed by fluorescence microscopy [44, 45]. Apoptosis was identified by the findings of condensation of chromatin and blebbing of the cytoplasm [45]. A total of five random microscope fields, each containing about 100 cells, were examined under each experimental condition. The total number of cells with histomorphological evidence of apoptosis was counted in each field.

4.5 Microinjection of DNA into MVEC

Construction of plasmids to express a FLAG tag attached to bcl-xL protein has been described [46]. MVEC were cultured in gelatin-precoated 3.5-cm diameter dishes for 2 days. Rectangles (10 by 3 mm) containing 150 to 200 mouse brain MVEC were drawn on the bottom of each dish to allow localization of injected cells. pSFFV-bcl-xL-plasmid or the empty pSFFV-plasmid solution at 100 ng/ml were injected into

MVEC nuclei with a mechanical Leitz manipulator and an INJECT + MATIC microinjector [46]. After 10 days in culture in the presence of medium with ECGS and 100 μ g/ml geneticin, the percentage of microinjected MVEC expressing FLAG-bcl-xL was determined by immunohistochemistry, using M2, an mAb specific for FLAG (International Biotechnologies), followed by FITC-conjugated rat-anti-mouse IgG. Greater than 80 % of the MVEC microinjected were positively stained. Cells microinjected with the control vector survived the antibiotic treatment, while control cells were all killed after 10 days in the presence of 100 μ g/ml geneticin.

4.6 Cell-based ELISA

MVEC cells (3×10^5 cells/ml) were left to adhere for 12 h in 96-well plates (Falcon) in DMEM + 20 % FCS. The plate was then washed once with HBSS, 20 mM Hepes (= washing buffer), after which the cells were fixed for 45 min at room temperature with DMEM + 1 % paraformaldehyde. After one washing step, the cells were incubated for 45 min at room temperature in blocking solution (PBS + 5 % BSA + 0.02 % NaN_3).

Subsequently, the wells were aspirated and 30 μ l of either a polyclonal rabbit-anti-mTNFR1 or a rabbit-anti-mTNFR2 [47] was added for 45 min at room temperature. To assess non-specific binding, the reactivity of rabbit-anti-mTNFR1 with MVEC from *Tnfr1*^o and of rabbit-anti-mTNFR2 with MVEC from *Tnfr2*^o was measured. After two washing steps, 50 μ l/well of a goat anti-rabbit-alkaline phosphatase complex (Biogenex, 1 μ g/ml) was added for 75 min under smooth agitation at room temperature. Finally, after two washing steps with washing buffer and one washing step with 2.5 M diethanolamine buffer, pH 9.5, 100 μ l/well of the substrate solution, consisting of the substrate Attophos™ (Europa Research Products, Ely, GB) (final concentration 1 mM) was diluted in 2.5 M diethanolamine buffer, pH 9.5, to which levamisole (an inhibitor of endogenous alkaline phosphatase, final concentration 10 mM) was added. After 2 min of incubation, the plate was read at 485 nm excitation wavelength and 530 nm emission wavelength in a Cytofluor® II fluorescence multi-well plate reader (PerSeptive Biosystems).

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