TNF in Septic Shock and Cerebral Malaria

R. Lucas, P. M. Suter, and G. E. Grau

Introduction

A massive production of inflammatory cytokines, such as tumor necrosis factor (TNF), interleukin-1 (IL-1) and interferon-γ (IFN-γ) occurs in conditions such as sepsis and infectious diseases. This cytokine overproduction can have deleterious effects on various organs, including lungs, liver or brain. One of the major target cells of inflammatory cytokines is the microvascular endothelial cell (MVEC), that is both morphologically and functionally different from large vessel endothelial cells (Table 1). Apart from being sensitive towards the direct cytotoxicity of some of the inflammatory cytokines such as TNF, activated MVEC can also stimulate and attract leukocytes, via the production of chemokines, and regulate their extravasation into tissues. We will review the direct and indirect effects of TNF on endothelial cells that can be relevant for the pathogenesis of septic shock, with particular attention to acute respiratory distress syndrome (ARDS), and cerebral malaria.

ARDS develops in 20–50% of patients after severe disturbances such as multiple trauma, sepsis, or shock [1]. Mortality rate appears to decrease over the last 10 years, but is still around 40% [2]. The pathogenic mechanisms of ARDS remain incom-

Table 1. Some functional differences between large vessel endothelial cells (LVEC) and microvascular endothelial cells (MVEC)

	LVEC	MVEC	Author, date	Ref.
Organ specific antigens	−	+	Auerbach et al., 1985	[56]
CD36	−	+	Swerlick et al., 1992	[57]
CD44	+	−	Swerlick et al., 1991	[58]
ICAM-1				
baseline	low	high	Wong and Dorovinizis, 1992	[39]
response to cytocines	weak	high	Swerlick et al., 1991	[38]
response to LPS	high	weak	Detmar et al., 1992	[59]
VCAM-1				
response to IL-1 or IL-4	+	−	Swerlick et al., 1992	[47]
persisting time	>72 h	<48 h	Lee et al., 1992	[60]
E-selectin				
response to TNF	short	long	Haraldsen et al., 1996	[41]
response to LPS	long	short		

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pletely understood. This syndrome is characterized by an activation of intrapulmonary and circulating cells, such as monocytes, macrophages and neutrophils, resulting in the massive release of cytokines. Indeed, elevated concentrations of TNF, IL-1β [3, 4] and IL-8 [5–9] are detectable in broncho-alveolar lavage fluids but not in the serum of ARDS patients. There is also good evidence that vascular endothelial cells are implicated in ARDS pathogenesis [10].

In systemic inflammatory diseases such as sepsis or septic shock, a pan-endothelial injury seems to take place [11]. ARDS results from an endothelial dysfunction that develops more particularly in pulmonary microvessels, involving endothelial-leukocyte interactions. Mechanisms of rolling, adhesion and migration of neutrophils on and through the surface of endothelial cells layers have been described (reviewed in [12]) and are thought to represent important steps in cell activation, changes in endothelial permeability, and pulmonary edema. Rolling of leukocytes is mediated by members of the selectin family, including L-selectin (LECAM-1, Mel-14, CD62-L) expressed on neutrophils, as well as E-selectin (ELAM-1, CD62-E) and P-selectin (CD62-P) expressed on endothelial cells. On the other hand, firm adhesion and extra-vascular migration of leukocytes appear to require β-2 integrins, leukocyte-function artigen 1 (LFA-1, CD11a/CD18), and Mac-1 (CD11b/CD18), and their endothelial receptors, intercellular adhesion molecules (ICAM) 1 and 2, belonging to the immunoglobulin superfamily. Vascular adhesion molecule-1 (VCAM-1) serves as a receptor for the integrin, very late antigen-4 (VLA-4). In addition to the binding of lymphocytes, VCAM-1 may also participate to neutrophil adherence (reviewed in [13]). It has been shown that CD18, the β chain of the β-2 integrins, plays a crucial role in thromboxane A2-induced neutrophil adhesion to endothelial cells [14]. Elevated CD11b/CD18 expression on circulating leukocytes has been described following post-traumatic ARDS [15], but adherence of neutrophils has been found either reduced [16] or enhanced [17] in patients with ARDS. Surface molecules such as TNF receptors p55 and p75 (TNFR1 and TNFR2) [18], or soluble CD14 [19] may also have a role in endothelial changes. The type of endothelial cell activation differs in acute versus chronic inflammation, as evidenced by the pattern of cell adhesion molecules induced or upregulated on their surface: for instance, acute *E. coli* infusion is associated with widespread E-selectin expression, while chronic lung injury following sepsis is rather characterized by ICAM-1 or VCAM-1 upregulation [20].

Malaria still represents the world's most important parasitic disease, and is responsible for the death of more people than any other communicable disease, except tuberculosis. According to World Health Organization estimations [21], the prevalence of the disease is between 300 and 500 million people infected by malaria every year. The disease is a public health problem in more than ninety countries, inhabited by a total of some 2400 million people, 40% of the world's population. More than 90% of all malaria cases are in Sub-Saharan Africa. Mortality due to malaria is in the range of 1.5 to 2.7 million deaths per year. Deaths occur mostly among young children in Africa, especially in remote rural areas with poor access to health services. Cerebral malaria (CM) is the most severe complication and major cause of death. In some reports, CM accounts for up to 10% of all cases of *Plasmodium falciparum* malaria admitted to hospital and for 80% of fatal cases. The classic clinical presentation of malaria consists of bouts of fever accompanied by other symptoms such as

headache, malaise, nausea, muscular pains, or mild diarrhea, often mistaken for influenza or gastrointestinal infection. Details of diagnosis, outcome per different types of patients, and treatment recommendations have been extensively reviewed [22].

Although various hypotheses have been proposed and some progress has been made using *in vitro* as well as *in vivo* models, the mechanisms of CM pathogenesis remain incompletely understood. Experimental models in animals cannot reproduce all the features of human diseases, and this is particularly true in the case of cerebral malaria. However, CM is a syndrome in which the same altered cell (the brain MVEC) has a pivotal role in human and experimental lesions; in addition, the similarities existing between defined malaria antigens in rodent and human parasites and between pathways of immune responses in mouse and human justify the use of a model. The concepts defined in experimental conditions might orientate further investigations in the human models. Both murine and human CM are characterized by TNF overproduction [23, 24]. TNF and IFN-γ are powerful inducers of adhesion molecules on endothelial cells, such as ICAM-1, VCAM-1 and ELAM-1. The implication of these adhesion molecules in experimental CM, a syndrome characterized by the development of brain lesions upon infection by *Plasmodium berghei* ANKA (PbA), was investigated by *in vivo* treatment with neutralizing monoclonal antibodies. As such, it was found that the ICAM-1/LFA-1 interaction is crucial for the development of the brain lesions [25, 26]. The ICAM-1 upregulation on the vascular endothelium in the brain occurs in both murine and human CM [25–28] and correlates with an increased trapping of leukocytes (murine CM) and of parasitized erythrocytes (model of human CM in SCID mice) [29].

TNF, a pleiotropic cytokine mainly produced by activated macrophages and T cells, induces hemorrhagic necrosis of certain tumors *in vivo* [30]. This effect was shown to be at least partially mediated by the TNF-induced endothelial injury of the tumor microvasculature [31, 32]. Therefore, MVEC represent essential target cells of TNF's necrotic activity. TNF binds to at least two types of receptors, TNFR1 of 55 kD, and TNFR2 of 75 kD, that are expressed on most somatic cells, except erythrocytes and unstimulated T cells (reviewed in [18]). Most *in vitro* studies assessing the cytolytic activity of TNF on primary cells, such as hepatocytes[33], have indicated an exclusive role for TNFR1 in this phenomenon. *In vivo*, both TNFR1 [18, 34, 35] and TNFR2 knock-out mice [36], named TNFR1° and TNFR2° mice, respectively, were reported to display an increased resistance towards high TNF doses, indicating also an important role of TNFR2 in the systemic toxicity of TNF. Moreover, both TNFR1 and TNFR2 were shown to be involved in TNF-induced local skin necrosis [37]. The apparent discrepancy between the *in vitro* and the *in vivo* results regarding the respective role of both TNF receptors might be explained by the fact that *in vivo*, TNF not only exerts direct effects on its target cells, but also indirect effects, such as the induction of adhesion molecules, that increase the interaction of these cells with leukocytes. Taken together, these results indicate that for most of the investigated TNF-mediated pathological situations, there is a critical role for TNFR1, whereas the role of TNFR2 remains unclear.

Purification of MVEC from Diseased Tissues

MVEC differ both phenotypically and functionally from large vascular endothelial cells (LVEC) (Table 1). Since MVEC also show organ specificity, one should preferentially isolate them from the diseased tissue one wishes to study. So far, dermal [38], cerebral [39], pulmonary [40], intestinal [41], and synovial [42] MVEC have been successfully isolated from human tissues. In order to study phenotype and functional changes induced in MVEC during disease, we have isolated lung MVEC from patients with ARDS and brain MVEC from mice susceptible to cerebral malaria [43, 44].

Phenotypic Changes in Pulmonary MVEC Purified from ARDS Patients

The questions we initially addressed were: 1) does pulmonary endothelial cell phenotype differ in septic shock? and 2) does endothelial responsiveness to cytokines differ in damaged versus normal tissues? To this end, pulmonary MVEC were isolated from the lung of patients who developed ARDS, and from patients who had undergone a lobectomy for lung carcinoma, as controls [43]. We assessed the expression of adhesion molecules and other surface molecules by flow cytometry, and the capacity to produce the cytokines IL-6 and IL-8 both constitutively and under TNF stimulation. The constitutive expression of ICAM-1 and, to a lesser extent, VCAM-1, was significantly increased on MVEC isolated from all ARDS patients, as compared with control MVEC. CD14 and TNFR2 were also increased on the surface of MVEC isolated from some patients with ARDS. The expression of ELAM-1 and TNFR1 was not significant on the surface of either ARDS-derived or control pulmonary MVEC. The constitutive ability of ARDS-derived MVEC to secrete IL-6 and IL-8 was markedly enhanced as compared with control MVEC. Upon *in vitro* re-stimulation by TNF, pulmonary MVEC from ARDS patients showed lower ICAM-1 upregulation, but similar IL-6 and IL-8 production capacity, when compared with control MVEC (Table 2). Thus, selective differences were found in cell adhesion molecules and TNFR2 expression on pulmonary MVEC isolated from patients with ARDS. These pulmonary MVEC spontaneously overexpress some adhesion molecules and produce greater amounts of the pro- and anti-inflammatory cytokines IL-8 and IL-6. These findings suggest that ICAM-1 and TNFR2 may have a particular involvement in the pathogenesis of acute lung injury, and that the endothelium may be an important source of cytokines detected in broncho-alveolar lavage during this syndrome [43].

We also compared the hemostatic properties of pulmonary MVEC to those expressed by their brain counterpart [44]. In addition, we extended the functional analysis of ARDS-derived MVEC by characterizing their hemostatic pattern. To address these questions, MVEC from human lung and brain were isolated and stimulated with TNF and *E. coli* lipopolysaccharide (LPS) for 24 h. The level and the kinetics of procoagulant activity (PCA) and thrombomodulin (TM) expression were found to be different depending on the tissue of origin and on the agonist used. In particular, the inducible PCA was higher in lung than in brain MVEC, an observation that may be related to the frequency of lung damage in septic shock. Differences were

Table 2. Phenotypic and functional changes observed in pulmonary MVEC derived from ARDS patients

	Surface expression or activity compared to control cells
Phenotypic variables	
ELAM-1	—
ICAM-1	↑↑↑
VCAM-1	—
TNFR1	—
TNFR2	↑↑
CD14	↑
Functional variables	
IL-6 production[a]	↑
IL-8 production[a]	↑
adhesiveness for leukocytes	↑
sensitivity to TNF-induced killing	↓

[a] both constitutive and TNF-induced (From [43])

also observed for tissue plasminogen activator (t-PA) and plasminogen activator inhibitor 1 (PAI-1) with MVEC supernatants or cell lysates. These variables were then measured in lung MVEC purified from patients with ARDS and compared to controls. Cells from ARDS patients constitutively expressed more PCA and PAI-1 than controls. The fibrinolytic potential, expressed as t-PA/PAI-1 ratio, was lower in ARDS than in lung MVEC. It is concluded that MVEC display different hemostatic features depending on the tissue they come from and that lung MVEC from ARDS patients present a procoagulant profile when compared with those from controls [44].

Our results raise the question whether the differences observed are due to either a genetic predisposition to ARDS based on MVEC phenotype or to a long-lived MVEC phenotypic change induced by ARDS. By allowing the monitoring of phenotypic and functional parameters, cultures of pulmonary MVEC isolated from ARDS patients may thus represent a useful system to analyze further the mechanisms of acute lung injury and to evaluate the efficacy of drugs, including inhibitors of cytokines and of adhesion molecules.

Functional Changes in MVEC from ARDS Patients

Among the various phenotypic changes seen in pulmonary MVEC isolated from ARDS patients, we focused our attention on the upregulation of TNFR2 [43]. We next evaluated the possibility that TNFR2 upregulation in human lung MVEC is associated with an altered susceptibility towards the combined lytic activity of TNF and the transcriptional inhibitor actinomycin D, a combination that was shown to efficiently lyse hepatocytes [33]. Under these conditions, MVEC were more sensitive towards TNF cytotoxicity than LVEC.

Furthermore, we compared ARDS patient-derived MVEC with high or low TNFR2 expression for their sensitivity to TNF-induced lysis. ARDS-derived MVEC expressing high levels of TNFR2 are less sensitive towards TNF-induced lysis than their

counterparts isolated from control patients. Interestingly, when pretreating MVEC *in vitro* with LPS, this results in an upregulation of TNFR2 and a decrease in sensitivity towards TNF and actinomycin D. Therefore, it seems possible to reproduce *in vitro* disease-associated functional changes of MVEC.

Does TNFR2 Have a Role in Direct TNF Cytotoxicity?

Since transcriptional inhibition of various cell types can dramatically change or even induce their responsiveness to TNF, we preferred to assess the cytotoxic effect of TNF on MVEC in the absence of sensitizing agents, to be as close as possible to physiological conditions.

Lung MVEC have been reported to be resistant towards the direct lytic activity of TNF, in contrast to LVEC, which various groups found to undergo apoptosis in response to TNF [45, 46]. On the other hand, *in vivo* observations have revealed that MVEC are more sensitive to TNF than LVEC following systemic infusion [47]. In order to investigate this apparent discrepancy between the *in vivo* and the *in vitro* findings, we first identified the conditions under which TNF kills MVEC. Confluence was found to be required to detect MVEC killing by TNF.

Second, using MVEC isolated from TNFR1 and TNFR2 gene-deficient mice, we investigated the respective role of each TNF receptor in TNF-induced killing, in the presence or absence of sensitizing agents. In the presence of Act. D, TNFR1 is necessary and sufficient to mediate apoptosis of MVEC. This result is in agreement with the reported resistance of hepatocytes isolated from mice lacking the TNFR1 [33]. In sharp contrast, in the absence of sensitizing agents, both TNFR1 and the TNFR2 were required for TNF-induced apoptosis to occur on lung MVEC. Indeed, cells isolated from either TNFR1°- or TNFR2°-mice were resistant, whereas cells isolated from wild type mice were lysed, as shown in Table 3. Thus, depending on the presence or absence of transcriptional inhibitors, different pathways can be implicated in the TNF-mediated 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 [48].

In conclusion, our results show that proliferating MVEC are not killed by TNF alone, unless they are treated with transcriptional inhibitors. This sensitized TNF lysis is mediated by TNFR1. In contrast, growth-arrested MVEC undergo apoptosis in

Table 3. Respective role of both TNF receptors in TNF-mediated cytotoxicity on lung MVEC. (From [49] with permission)

		Response to TNF	
Origin of MVEC	TNFR present	with Act. D	without Act. D
wt B6x129	R1 + R2	killed	killed
TNFR1°	R2	resistant	resistant
TNFR2°	R1	killed	*resistant*

response to TNF itself, and this phenomenon requires the presence of both receptors, since it is blocked by either TNFR1- or TNFR2-deficiency.

Is TNFR2 Implicated in TNF-Mediated Pathology?

As in pulmonary MVEC derived from ARDS patients, the only TNFR-type found upregulated in brain microvessels during CM was TNFR2. This increase in TNFR2 expression only occurred in CM-susceptible mice, at the onset of the neurological syndrome. We therefore investigated the role of TNFR2 in the development of this brain pathology, by comparing the incidence of CM in wild type B6x129 mice, TNFR1°- [34] and TNFR2°-mice [36]. Unexpectedly, the genetic deficiency in TNFR2, but not in TNFR1, conferred protection against CM and its associated mortality [49]. The evolution of parasitemia, however, was identical in the two types of knock-out animals and in the wild type mice.

In view of this essential role of TNFR2 in experimental CM, the mechanism by which these mice are protected was investigated. Compared to non-infected mice, all mice presented the same rise in serum TNF and IFN-γ levels. Thus, the protection of TNFR2°-mice cannot be explained by a decrease in serum concentrations of critical mediators of CM. In agreement with our findings in lung MVEC, brain MVEC isolated from wild type mice were efficiently lysed by TNF, whereas cells isolated from both TNFR1°- and TNFR2°-mice were resistant to direct TNF cytotoxicity. However, this result in itself cannot explain why only TNFR2°-mice are significantly protected from CM. Therefore, we evaluated the possible implication of TNFR2 in ICAM-1 upregulation.

The increase of ICAM-1 expression on brain MVEC is indeed of crucial importance for the subsequent brain damage, since it increases the interactions between the brain endothelium, sequestering platelets and leukocytes [50]. Therefore, we assessed ICAM-1 expression on brain microvessels of wild type, TNFR1°- and TNFR2°-mice. Brain microvessels of PbA-infected wild type and TNFR1°-mice, both of which developed CM, showed a significant increase in ICAM-1 expression. In sharp contrast, no ICAM-1 upregulation could be detected in the brain of TNFR2°-mice, indicating a close correlation between protection against CM-associated brain damage, absence of TNFR2 and absence of ICAM-1 upregulation in the brain.

Our current results provide evidence for a cerebral upregulation of TNFR2, and for its subsequent involvement in CM-associated brain damage. A similar upregulation of TNFR2 has been observed on hepatocytes from chronic hepatitis B patients [51] and, as discussed above, on lung MVEC from ARDS patients [43].

The mechanism by which TNFR2°-mice are protected from CM correlates with the crucial role of TNFR2 in TNF-mediated ICAM-1 upregulation in brain microvessels. This result is surprising, since most *in vitro* studies using endothelial cells have shown that TNFR1, rather than TNFR2 is implicated in ICAM-1 upregulation induced by soluble mouse TNF [52]. In spite of the crucial role of TNFR1 in the induction of ICAM-1 by soluble TNF, TNFR1°-mice show an increased expression of this adhesion molecule on brain MVEC upon malaria infection. On the other hand, it is remarkable that TNFR2°-mice do not show ICAM-1 upregulation in the brain, even though they express normal TNFR1 levels and have high serum TNF levels (Fig. 1).

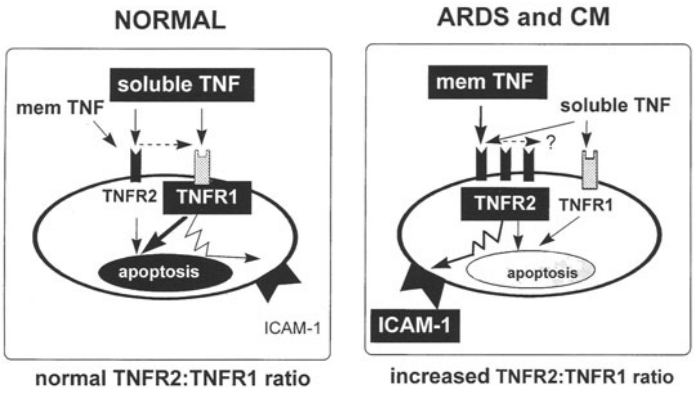

Fig.1. Central role of TNFR2 in endothelial changes of septic shock/ARDS and cerebral malaria. In normal conditions, TNFR1 is primarily implicated in apoptosis and ICAM-1 induction by soluble TNF. In inflammatory conditions, occuring during septic shock/ARDS and CM, TNFR2 upregulation leads to decreased sensitivity towards soluble TNF and to increased sensitivity towards membrane-bound TNF-induced ICAM-1 induction, mainly mediated by TNFR2.
MemTNF: membrane-bound TNF; *ARDS:* acute respiratory distress syndrome; *CM:* cerebral malaria; *TNFR1:* TNF receptor 1; *TNFR2:* TNF receptor 2

In order to explain the apparent discrepancy between the *in vivo* and *in vitro* results regarding TNF-induced ICAM-1 upregulation, we propose that, rather than soluble TNF, the membrane form (26 kD) of the cytokine may have a role in CM, since it was recently suggested to preferentially interact with TNFR2 [53] and does not undergo ligand passing from TNFR2 to TNFR1 [54, 55] (Fig. 1). Since CM is associated with a selective upregulation of TNFR2 in brain microvessels, it increases the TNFR2/TNFR1 ratio on brain MVEC. As a consequence, the ligand passing of soluble TNF from TNFR2 to TNFR1 may be less efficient, in view of the proportionally insufficient amount of TNFR1. In contrast to soluble mouse TNF, membrane-bound TNF, present on sequestering leukocytes and possibly also on astrocytes during CM, might be able to induce ICAM-1 expression on brain MVEC by means of TNFR2, thereby providing an explanation for the critical role of this TNF receptor in CM.

Taken together, our results provide the first evidence for an important role of TNFR2 in CM-associated ICAM-1 upregulation in the brain. The discrepancy between the *in vitro* and *in vivo* results regarding the role of the two TNF receptor types in the ICAM-1 induction in the brain MVEC could indicate an important role for membrane-bound TNF in this process.

Conclusion

The observation of constitutive alterations in lung MVEC isolated from ARDS patients prompts the question of a genetic predisposition based on an endothelial phenotype or of disease-induced changes. Our results in ARDS and CM indicate a specific upregulation of TNFR2, but not of TNFR1, on lung and brain MVEC, respectively. This increased expression leads to a decreased sensitivity towards TNFR1-

mediated phenomena, such as the sensitised TNF cytolytic activity. In contrast, the sensitivity towards TNFR2-mediated effects, such as ICAM-1 induction by membrane-bound TNF, is increased on these cells. Therefore, the ICAM-1 inducing effect, rather than the direct cytotoxicity of inflammatory cytokines, such as TNF, appears to be crucial in sepsis and CM-induced endothelial damage, and TNFR2 seems to play an important role in this activity *in vivo*.

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