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TNF: a moonlighting protein at the interface between cancer and infection

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1. ABSTRACT

The remarkable ability of TNF, especially in combination with Interferon-gamma or melphalan, to inhibit the growth of malignant tumor cells is so far unmatched. Unfortunately, its high systemic toxicity and hepatotoxicity prevent its systemic use in cancer patients. An elegant manner to circumvent this problem is the isolated limb and liver perfusion for the treatment of melanoma, soft tissue sarcoma and liver tumors, respectively, although the latter method can lead to a reversible hepatotoxicity. In order to allow also the treatment of other cancers with TNF, new strategies have to be developed that aim at sensitizing tumor cells to TNF and at reducing its systemic and liver toxicity, without losing its antitumor efficiency. Moreover, the lectin-like domain of TNF, which is spatially distinct from the receptor binding sites, could be useful in reducing cancer treatment-related pulmonary edema formation. This review will discuss some recent developments in these areas, which can lead to a renewed interest in TNF for the systemic treatment of cancer.

2. INTRODUCTION

The Egyptian physician, architect and priest Imhotep proposed around 2600 BC to make an incision, which inevitably lead to a local infection, inflammation and fever, as recommended treatment for swellings (the nomination for tumors in those days) (1). Moreover, in 500 BC, the greek philosopher Parmenides claimed: "Give me the power to induce fever and I shall cure every illness". In 1868 in Germany, Dr. Busch intentionally infected a patient with a soft tissue sarcoma of the neck with erysipelas, hoping that it would cause a tumor regression. After being infected with erysipelas (*Streptococcus pyogenes*), rapid tumor shrinkage was indeed observed, but this response was only partial and tumor recurrence subsequently occurred (2, 3). As such, the fever therapy was born, although it was far from being optimized. A more systematic approach was undertaken by US surgeon William B. Coley (active career 1891-1936), who first infected ten cancer patients with erysipelas, leading to high fever. Due to the unpredictability of this treatment, ranging from poor to

fatal infection, he improved this treatment by means of using a bacterial vaccine ("Coley's toxins"), containing two types of killed bacteria, i.e. *Streptococcus pyogenes* and *Serratia marcescens*. As such, using a therapy simulating an infection (fever, inflammation), around 900 patients with primarily inoperable sarcoma were treated, causing a remarkable cure rate of better than 10% (4). Later on, these Coley's toxins were also successfully used for the treatment of carcinomas, lymphomas and myelomas (5). The scientific explanation of the success of the fever therapy started to be unravelled by Dr. Shear's research group (USA), who first reported in 1936 that lipopolysaccharide (LPS) from Gram-negative bacteria efficiently induced tumor necrosis, and then discovered around 1960 that not LPS itself, but rather a protein induced by LPS was responsible for this activity (6). Dr. Old's research group was able to characterize this protein in 1975, which could be isolated as a serum factor, effective in causing hemorrhagic necrosis of a methylcholanthrene-induced sarcoma *in vivo,* derived from mice infected with Bacillus Calmette-Guérin (BCG) and treated with LPS. This serum factor, which was called Tumor Necrosis Factor (TNF) (7), was then cloned by several research groups at the beginning of the 80's in Belgium, Japan and the US (8-10). Human TNF is a non-glycosylated, non-covalently linked homotrimeric protein of 51 kD, generated as a membrane-bound precursor (11), that is cleaved by the TNF-alpha Converting Enzyme (TACE) (12), which belongs to the family of the metalloproteinases (ADAM 17: A Disintegrin And Metalloprotease 17), as such giving rise to the soluble protein. Interestingly, apart from TACE, also proteases, as e.g. proteinase 3, mainly produced by neutrophils, were shown to be able to cleave membrane-bound TNF, in order to generate soluble TNF (13-15). Although many cell types are able to produce TNF, the main source of the cytokine are activated macrophages and T cells. TNF binds to two different TNF receptors, TNF-R1 (55 kD) and TNF-R2 (75 kD), at least one of which is expressed in most somatic cells (16, 17). The receptor binding sites of TNF are situated at the interface between neighbouring subunits, so that every TNF trimer can bind to three receptors (18, 19). As such, the cross-linking of the receptors with agonistic antibodies is able to mimic TNF signalling (20). Soluble TNF was proposed to have the highest affinity for TNF-R1 (TNF-R1: Kd = 1.9 x 10^{-11} M; TNF-R2: Kd = 4.2 x 10^{-10} M), whereas membrane-bound TNF was proposed to preferentially interact with TNF-R2 (21-23). Like TNF, also the receptors exist in a membrane-associated and soluble form, the latter of which corresponds to the extracellular part of the receptors and is cleaved by the same enzyme cleaving membrane-bound TNF (i.e. TACE) (24, 25). At high concentrations, the soluble TNF receptors can neutralize most TNF activities. Moreover, in view of the short half life of TNF (less than 20 min in human serum), these soluble receptors were shown not only to neutralize all TNF receptor-mediated activities, but also to stabilize TNF structure, thus preserving its activity and augmenting some of its effects (26).

3. RESULTS

3.1. Tumor *necrosis* factor: what's in a name?

The so-called "necrotic" activity of TNF refers to the effects of the cytokine on the entire tumor mass, rather than to the effects on the individual tumor cells. The TNF-mediated hemorrhagic necrosis of certain tumors *in vivo* is mainly caused by the destruction of the tumor microvasculature. Interestingly, TNF mostly kills endothelial cells as well as most other TNF-sensitive cells by means of apoptosis, rather than by necrosis. Indeed, TNF-R1 is a classical death receptor, thus expressing a death domain, which upon aggregation will bind to the TNF Receptor Associated Death Domain (TRADD). TRADD will then recruit RIP as well as the Fas Associated Death Domain (FADD), which will then associate to procaspase 8 (27, 28). Procaspase 8 will then undergo cross-activation through induced proximity, as such generating the active initiator caspase 8. Caspase 8 will subsequently cleave an effector caspase, such as caspase 3, which will then induce apoptosis. Many cells are protected from the TNF-R1 mediated apoptosis, since they express high levels of anti-apoptotic proteins, such as c-FLIP and members of the IAP family (29). Moreover, in the presence of high concentrations of the TNF Receptor Associated Factor 2 (TRAF 2), the Death Inducing Signaling Complex (DISC) will not be activated (30). In contrast, under those conditions, TNF will induce NF-kappaB activation, as such activating the expression of pro-inflammatory genes. In contrast to TNF-R1, TNF-R2 does not contain a death domain (28). Therefore it could be assumed that this receptor is not implicated in apoptosis. However, this assumption seems to be wrong, in view of several observations: i) although HeLa cells, which mainly express TNF-R1, are not killed by hTNF, the overexpression of TNF-R2 makes them sensitive to hTNF-induced apoptosis (31), ii) TNF-R2 was shown to be able to induce apoptosis in the transfected rat/mouse T cell hybridoma PC60, independent of TNF-R1 (32), and iii) confluent murine brain and lung microvascular endothelial cells (MVEC) isolated from both TNF-R1$^{-/-}$ or TNF-R2$^{-/-}$ mice are protected from mouse TNF-induced apoptosis, whereas cells from wild type animals die upon treatment with mouse TNF (33). The latter result that microvascular endothelial cells can be directly killed by TNF, is different from what was reported by others (34, 35). However, those studies, claiming that TNF can only kill endothelial cells in the presence of sensitizing agents or in the presence of synergistic cytokines, such as Interferon-gamma, have mainly used large vessel endothelial cells, which are morphologically and phenotypically distinct from the microvascular endothelial cells (36). Under sensitized conditions, only TNF-R1 was proposed to mediate apoptosis, which we could confirm also in MVEC (33). We moreover found that the overexpression of the anti-apoptotic protein bcl-xL protected the MVEC from direct, but not from sensitized TNF-induced apoptosis, indicating a link between the activation of TNF-R2 and the expression of bcl-xL, as was also proposed by others in T lymphocytes (37). Another mechanism by which the activation of TNF-R2 can influence apoptosis consists in the

ubiquitinylation and subsequent destruction of TRAF-2, resulting in an increased activation of the TNF-R1 induced apoptotic pathway (38). In 1990, Dr. Clauss and colleagues discovered a 44-kDa polypeptide in the supernatant of Meth-A fibrosarcoma cells, which was later called Endothelial Monocyte Activating Polypeptide II (EMAP II). Interestingly, EMAP II increases the expression of TNF receptors on endothelial cells (39, 40) and thus likely enhances the effect of TNF on the tumor-associated microvasculature. Moreover, EMAP II was also shown to augment the TNF sensitivity of TNF-resistant tumors (41). Recently, potential additional anti-tumor effects of EMAP II, when used in combination with TNF in the ILP setting were shown (42). Upon combined TNF/IFN-gamma treatment in ILP, a significantly reduced activation of the integrin alphaVbeta3, which is critically involved in mediating tumor angiogenesis, occurs, leading to a detachment and apoptosis of angiogenic endothelial cells *in vivo* in melanoma metastases of patients. This thus provides a possible mechanism of TNF's potential to disrupt the tumor vasculature (43).

3.2. Strategies to increase TNF sensitivity and availability in tumor cells

Most tumor cell types are resistant towards TNF-induced cytotoxicity, unless they are transcriptionally or translationally inhibited. It was previously shown that lithium salts significantly increase the direct cytotoxic effect of tumor necrosis factor (TNF) on various tumor cells *in vitro* and *in vivo*, independent of the type of cell death. In the case of apoptosis, the TNF/lithium synergism involves an enhanced caspase activation and an increase in mitochondrial cytochrome c release. Interestingly, the LiCl-induced sensitization to apoptosis seems to be specific for TNF-induced apoptosis, since CD95 ligand- or etoposide-induced apoptosis remain unaffected. (44). Recently, interesting new strategies have been developed in order to increase the TNF-sensitivity of tumor cells. Indeed, inhibitor of apoptosis (IAP) proteins, which are anti-apoptotic regulators blocking cell death in response to diverse death receptor stimuli, including TNF, are expressed at increased levels in many human tumor cells and thus represent attractive targets for the development of novel cancer therapeutics. Small-molecule IAP antagonists were shown to bind to select baculovirus IAP repeat (BIR) domains, leading to a dramatic induction of auto-ubiquitination activity and a rapid proteasomal degradation of c-IAPs (45). Interestingly, these IAP antagonists themselves were shown to induce cell death in the treated tumor cells, by means of inducing de novo TNF synthesis in these cells (45-47). Additionally, the c-IAP proteins were found to function as regulators of NF-kappaB signaling, since through their ubiquitin E3 ligase activities, c-IAP1 and c-IAP2 promote proteasomal degradation of NIK, the central ser/thr kinase in the non-canonical NF-kappaB pathway (48). Also XIAP activity, which prevents apoptosis by binding to and inhibiting caspases, can be relieved by IAP antagonists, such as Smac/DIABLO. Although XIAP inhibits post-mitochondrial caspases, small molecule IAP antagonist-induced apoptosis was shown to be blocked by a caspase 8 inhibitor (46). Also, tumor cells, upon c-IAP1 inhibition or deletion, became sensitive to apoptosis induced by exogenous TNF. An alternative approach to increase TNF sensitivity of tumor cells consists in the development of TNF prodrugs, which become processed by tumor and/or stroma-associated proteases, having *e.g.* a urokinase-type plasminogen activator (uPA)-selective or a dual uPA-MMP-2-specific linker. Such prodrugs containing a multi-specific protease linker were proposed to have a functional superiority over the wild type molecule (49). The processing of optimised TNF prodrugs should therefore increase the intratumoral active therapeutic, as such potentially enhancing the tumor response rate. Taken together, these results represent an important breakthrough in order to improve sensitivity towards TNF-induced apoptosis in tumor cells (50).

3.3. Strategies to prevent the systemic toxicity of TNF, while preserving its potent anti-cancer effects

The remarkable ability of TNF, especially in combination with Interferon-gamma, to inhibit the growth of malignant cell lines is so far unmatched by any other combination of cytokines. Unfortunately, clinical trials in cancer patients have been very disappointing, since the effective TNF dose exceeds the maximum tolerated dose by a factor of about 20 (51). Therefore, in spite of its antitumor properties, TNF currently cannot be used for the systemic treatment of cancer patients. It is somewhat surprising that the initial interest in TNF as a systemically applicable potential anti-cancer agent, largely ignored the important side effects already recorded with the fever therapy, such as hypotension and liver toxicity, the former of which was revealed in clinical trials to be the major dose-limiting factor. Hypotension was suggested to be the consequence of excessive nitric oxide (NO) production and subsequent soluble guanylate cyclase (sGC)-mediated vascular smooth muscle relaxation. In mice, large bolus injections of TNF provoke a lethal shock syndrome, with a cardiovascular collapse induced by excessive nitric oxide (NO) production. Surprisingly, NO synthase (NOS) inhibitors aggravated, rather than ameliorated outcome in animal sepsis models and septic shock patients, suggesting a bivalent role for NO. Indeed, apart from inducing lethal hypotension, NO also has an important anti-oxidant function, as such protecting affected organs from lipid peroxidation and oxidative stress. Apart from NO, also caspases were recently shown to exert unexpected and dual functions during TNF shock, in view of also exerting an important endogenous negative feedback on oxidative stress (52). Interestingly, TNF-induced hypotension was shown to be inhibited by methylene blue (MB), an inhibitor of the nitric oxide (NO)-induced activation of the cytosolic guanylate cyclase, leaving the indispensable protective properties of NO unaffected (53). Recent results also suggest that induction of heat shock protein 70 in mice, as can occur upon the addition of $ZnSO_4$ to the drinking water of mice, can protect them from the systemic toxicity of TNF, without interfering with the tumoristatic effect in a murine melanoma tumor model (54, 55).

3.4. Manipulating intra-hepatic ATP levels in order to reduce TNF-mediated hepatotoxicity

Whereas controlled hepatocyte apoptosis ensures homeostasis and adaptation of liver size, increased apoptosis rates upon overactivation of death receptors, such as CD95 and TNF-R1, can be observed during ischemia, transplant rejection, intoxication, viral infection, and autoimmune disease (56-59). The local application of TNF, preferentially in combination with the DNA cross-linking alkylating agent melphalan during isolated limb or hepatic perfusion (60-63) prevents random distribution of TNF in the circulation and thus systemic toxicity to a large extent. However, this treatment can be associated with TNF-mediated hepatotoxicity (63). Thus, a liver-selective control of TNF-induced hepatocyte death, while preserving the apoptotic activity of TNF towards liver tumor cells is a desirable goal to handle adverse reactions during therapeutic interventions in liver disease. The acute-phase proteins, alpha1-acid glycoprotein and alpha1-antitrypsin were shown to inhibit TNF-induced lethal hepatitis (64). An alternative means of reducing TNF's hepatotoxicity consists of manipulating the energy status of the hepatocyte. Indeed, the ATP level is pivotal for controlling apoptosis in different cell types (65, 66). Hepatocytes, in contrast to liver tumor cells, express high levels of the enzyme fructokinase, which rapidly converts fructose to fructose-1-phosphate, which in turn is slowly converted by the enzyme aldolase B to the glycolytic component fructose-1,6-biphosphate. The glycolytic substrate fructose has diverse effects in hepatocytes, depending on its concentration. At low concentrations it protects hypoxic hepatocytes from necrosis *via* ATP generation (67) and moreover inhibits apoptosis induced upon reoxygenation, by means of attenuating the generation of reactive oxygen species (68). In contrast, at high concentrations, fructose depletes ATP in hepatocytes (69, 70), but still leaves sufficient residual ATP (>15% of control) to avoid necrosis induction, observed after total ATP loss (71, 72). Extensive metabolic ATP depletion by fructose can protect primary murine hepatocytes from sensitized TNF-induced apoptosis *in vitro* and from high dose TNF-induced apoptosis *in vivo* (73). Since, apart from caspase-dependent apoptosis, TNF can also induce caspase-independent apoptotic, as well as necrotic cell death in the liver (74), we also assessed the role of ATP depletion in these models, which are all TNF-R1 dependent. As such, it was shown that fructose-mediated ATP depletion protected mice from high-dose LPS induced liver necrosis as well as from caspase-independent galactosamine/lipopolysaccharide (LPS)-induced apoptosis in the liver (75). These data thus indicate that TNF-R1 dependent hepatocyte death can be blocked by ATP depletion *in vitro* and *in vivo*. Our current understanding of the TNF-R1 signaling pathway is that after receptor engagement, the DISC is formed and subsequently caspase-8 is activated (76). From there on, two alternative pathways emerge: i) the caspase-3/-7 route or ii) the mitochondrial pathway *via* Bid cleavage, release of cytochrome c and its binding to Apaf-1 and pro-caspase-9 to form the apoptosome complex with ATP as an integral part (77). In primary murine hepatocytes, cell death is blocked in the ATP-depleted state upstream of apoptosome formation (73). The fructose-mediated protection against necrotic cell death puts a direct causal ATP-dependence of caspase activation or activity in question. Moreover, two events of TNF-R1 signaling, i.e. NF-κB activation and internalization of the TNF receptor are still functional under ATP depletion. It is therefore likely that the inhibition of TNF-mediated hepatocyte death upon metabolic ATP depletion is due to the incomplete DISC formation and signalling at the TNF-R1. The observation by others that a dominant negative FADD is sufficient to inhibit cell death without affecting NF-kappaB activation and gene induction upon TNF-R1 stimulation, supports this hypothesis (78). Many clinical series have reported overall response rates of 80–95% following isolation perfusion with TNF and melphalan for patients with in-transit melanoma metastases, unresectable soft tissue extremity sarcomas, or unresectable cancers confined to the liver (79). Interestingly, tumors with poor histological response to ILP with TNF and melphalan were shown to have significantly higher levels of p53-mutated protein, indicating an important role for the p53 protein status in the response to TNF and melphalan (80). The hepatic perfusion of melphalan, especially in combination with TNF, leads to a reversible hepatotoxicity in a significant amount of patients. We have observed that the antineoplastic drug melphalan can increase membrane TNF expression in Kupffer cells, since it is able to inhibit TACE (81). Moreover, by means of inducing transcriptional inhibition, the alkylating agent sensitizes hepatocytes to TNF mediated apoptosis. Interestingly, livers from either TNF-R1- or TNF-R2-deficient mice were shown to be resistant towards melphalan induced hepatotoxicity, whereas only livers from TNF-R1 deficient animals were protected by the combined treatment with melphalan and soluble TNF. This means that TNF-R2, although devoid of a death domain, is nevertheless implicated in melphalan-induced apoptosis. Interestingly, we could show that fructose, which leads to extensive ATP depletion, can protect hepatocytes from melphalan-induced apoptosis (81). Since the fructose-mediated ATP depletion only occurs in hepatocytes and not in liver tumor cells, this could thus represent an attractive means of protecting healthy hepatocytes from melphalan/TNF-mediated hepatotoxicity, while preserving the anti-neoplastic activities of the treatment (82).

3.5. TNF-R2: an unexpected player in leukocyte-endothelial interactions and inflammation

In accordance with its discovery at the interface between infection and cancer, it has become clear that TNF represents one of the most prominent cytokines involved in innate defence against bacterial (83, 84), fungal (86), viral (87) and parasitic infections (88-91). In most bacterial infection models, TNF-R1, rather than TNF-R2, was shown to be crucial for survival. Indeed, TNF-R1$^{-/-}$ mice were shown to be highly susceptible to infections with *Listeria monocytogenes* (83, 84), *Streptococcus pneumoniae* (92) and with mycobacteria and other granulomatous pathogens, both in the active and latent phases of infection (84, 93,

94). Long-term neutralization of TNF activity in patients suffering from rheumatoid arthritis, in spite of excellent effects, can lead to reactivation of tuberculosis in a part of the patient population (95). Moreover, immunosuppressed mice could only survive an otherwise lethal *Salmonella typhimurium* infection upon granulocyte-macrophage colony stimulating factor (GMCSF)-mediated restoration of TNF production (96, 97). However, when overproduced, as *e.g.* upon LPS-induced shock (98, 99) or during experimental cerebral malaria (100), the cytokine may lead to fatal multiple organ failure or brain damage, respectively. Thus, TNF represents a double-edged sword in innate immunity. Both experimental and human cerebral malaria are characterised by a sequestration of leukocytes and parasitized erythrocytes in the microvessels of the brain, finally causing fatal brain lesions. For the latter pathology, TNF-R2 was proposed to be crucial, since TNF-R2$^{-/-}$, but not TNF-R1$^{-/-}$ mice were shown to be resistant to *Plasmodium berghei* ANKA-induced cerebral malaria, and only brain microvascular endothelial cells isolated from TNF-R2$^{-/-}$ mice, but not those from wild type or TNF-R1$^{-/-}$ animals, were protected from both membrane-bound TNF and soluble TNF-induced ICAM-1 upregulation (101, 102). Indeed, TNF-R2 expressed on vascular cells (103) was proposed to be crucial for the TNF-mediated upregulation of ICAM-1, which interacts with its ligand LFA-1 expressed on leukocytes, as well as with the Duffy binding-like (DBL) domain, which is a key adhesive module in *Plasmodium falciparum* (104). ICAM-1$^{-/-}$ mice are moreover protected from Plasmodium berghei ANKA-induced cerebral malaria (105). Recently, TNF-R2 was proposed to be important not only in TNF-mediated ICAM-1, but also in VCAM-1 and E-selectin dependent leukocyte-endothelial interactions (106). Another mechanism by which TNF-R2 could contribute to cerebral malaria development is by means of metabolic disturbances linked to insulin sensitivity, selectively mediated by TNF-R2 (107). In accordance with an important role of TNF-R2 in ICAM-1 induction, we found that a mouse TNF mutant (T104A-E106A-E109A), which lacks the lectin-like activity of wt mTNF (see chapter 3.7), and which displays a significantly reduced TNF-R2, but not TNF-R1 bioactivity, has a significantly lower ICAM-1 and VCAM-1 inducing capacity and pro-metastatic activity, as compared to wt mouse TNF (82, 108). Although TNF is a major proinflammatory cytokine, it can also exert immunosuppressive feedback effects. Also in this activity, TNF-R2 seems to play an important role. Indeed, in both resting and activated states, mouse peripheral CD4+CD25+ T regulatory cells (Tregs) were shown to express significantly higher levels of TNF-R2 than CD4+CD25- T effector cells (Teffs). During cocultures of Tregs and Teffs, the former cells were shown to inhibit proliferation and cytokine production of the latter upon longer exposure to TNF (109). Furthermore, following sublethal cecal ligation and puncture the number of Tregs increased in wild-type mice, but not in TNF-R2$^{-/-}$ mice. These data thus indicate that the stimulatory effect of TNF on Tregs is even more pronounced because of the higher expression of TNF-R2 by Tregs (109).

3.6. TNF effects on tumor spreading and endothelial permeability

In spite of its potent tumoristatic activity, TNF was also shown to enhance tumor colony formation in different models of experimental and spontaneous metastasis (110-113). Different mechanisms can account for this activity. First, TNF was shown to impair Natural Killer Cell activity in tumor-bearing mice (113), which represents one means by which TNF supports tumor colony formation. Second, TNF was shown to activate the signaling of Focal Adhesion Kinase (FAK), a non-receptor protein tyrosine kinase that is frequently overexpressed in various tumors and whose expression shows a good correlation with tumor progression (114). FAK is involved in tumor spreading, proliferation, migration, and invasion. Moreover, the FAK signaling plays a critical role in the production of matrix metalloproteinases, such as MMP-2 and MMP-9, which play a crucial role in tumor extravasation (115). When however used in low concentrations, TNF, by means of enhancing the intratumoral accumulation of chemotherapeutic drugs upon manipulation of the tumor cell-stromal interface can make chemotherapy more effective (116). Apart from affecting tumor spreading, TNF was also shown to alter endothelial cell monolayer integrity (117), which represents a balanced system between contractile and tethering forces acting on endothelial cells. These forces depend on cytoskeletal components, including actin-based microfilaments, intermediate filaments and microtubules (118). The mechanisms by which TNF triggers endothelial barrier disruption occur by means of contracting the intracellular actin microfilaments and activating the formation of intercellular gaps, that parallel the development of transendothelial permeability (119). The rearrangement of the actin-based cytoskeleton is the result of an actin-myosin movement, which follows the phosphorylation of the myosin light chains. In large vessel endothelial cells, the mechanisms by which TNF triggers microtubule network disassembly, followed by actin cytoskeleton rearrangement and intercellular junction changes include the activation of the MAPK pathways. Each component of the MAPK pathways is differentially recruited by specific stimuli, resulting in kinase-specific signaling and regulation of cell growth, differentiation, and apoptosis. In pulmonary microvascular endothelial cells, the junctional permeability was proposed to be altered by mechanisms including protein kinase C (PKC) and p38 MAPK (120). Thus, TNF-mediated stimulation of the endothelium is often accompanied by enhanced cellular contraction and formation of intercellular gaps. Although these activities can be promising for improving delivery of chemotherapeutic agents into the tumor site, this response, when too intense, can also contribute to permeability edema, particularly in the lungs. Intriguingly, as will be discussed in the following chapter, TNF itself seems to harbor a substructure able to resolve this problem.

3.7. The lectin-like domain of TNF: the dark side of the moon

Although it is generally assumed that cytokines solely exert their activities upon activating their respective receptors, this does not seem to be completely true in the case of TNF. TNF, in contrast to Lymphotoxin-alpha, which has a highly homologous 3-D structure as TNF, and which is able to bind to both TNF receptors, was shown to exert a lytic activity in purified long slender blood stream forms of African trypanosomes (90, 121, 122). Interestingly, the trypanolytic effect of TNF on purified *T.brucei brucei* AnTat 1.1 parasites can not be inhibited by complexing the cytokine with the soluble TNF receptor 1, which blocks all TNF receptor-mediated effects (121). In view of the observation by others that TNF exerts a lectin-like activity, permitting its binding to glycoproteins such as uromodulin, a glycoform of Tamm-Horsfall protein found in the loops of Henle of pregnant women. Interestingly, uromodulin was shown to be able to bind the pro-inflammatory cytokines, IL-1-beta, IL-2 and TNF, proposed as a mechanism to clear excessive levels of these cytokines from the circulation during pregnancy (123, 124). Since uromodulin-bound TNF was still able to exert cytotoxic effects in L929 fibrosarcoma cells, it was proposed that the lectin-like domain of TNF has to be spatially distinct from its receptor binding sites. Our observations that i) specific oligosaccharides, such as *N,N'*-diacetylchitobiose, as well as branched trimannoses, which are known to bind to the lectin-like domain of TNF, are able to inhibit the trypanolytic, but not the cytotoxic activity of TNF in L929 cells, and that ii) lectins with a similar oligosaccharide specificity as TNF, such as Urtica Dioica Agglutinin, but not those with a differrent specificity, block the trypanolytic effect of TNF (121), lead to the hypothesis that the lectin-like domain of TNF is responsible for its trypanolytic activity. This was confirmed by the observation that TNF is able to bind to the Variant Surface Glycoprotein of the trypanosomes, upon which the cytokine is endocytosed and transported to the lysosomes, where intralysosomal rupture finally causes the trypanolysis to occur (125). Molecular graphics comparisons of tertiary structures of TNF (trypanolytic) and the highly homologous lymphotoxin-alpha (non-trypanolytic), lead us to propose a dissimilar structure that could be responsible for the lectin-like activity. This structure, which is present at the Tip of the TNF molecule could be mimicked by a circular 17 amino acid peptide, which we called the TIP peptide. Antibodies to this peptide were able to inhibit the trypanolytic activity and moreover, the TIP peptide itself was shown to exert trypanolytic activity (121). Three amino acids, i.e. one threonine and two glutamic acids were shown to be crucial for this activity. A Triple TNF mutant in which these three residues were mutated to alanines completely lacked trypanolytic activity, but nearly completely retained the TNF receptor 1-mediated protective effects of TNF in a ceacal ligation and puncture model, the anti-angiogenic activity of TNF in an *in vitro* endothelial morphgenesis assay, as well as the tumoristatic effects in combination with IFN-gamma in a murine B16BL6 melanoma murine model. These data thus indicate that a mutation in the lectin-like domain of the cytokine does not affect TNF receptor 1-mediated activities of the cytokine (108, 126). These mutations do however decrease TNF receptor 2 mediated activities, as indicated by its 50-fold reduced capacity to induce proliferation of CT6 cells, that mainly express TNF-R2, as compared to wt mouse TNF. This is possibly because of the proximity of the lectin-like domain of TNF to the receptor 2 binding site. Interestingly, apart from inducing trypanolysis, the lectin-like domain of TNF was also shown to activate sodium transport in alveolar epithelial cells, peritoneal macrophages and microvascular endothelial cells (127, 128). This is most probably not a direct effect of TNF, but rather involves the TNF-mediated activation of endogenous amiloride-sensitive sodium channels (129). Since the uptake of sodium in type II alveolar epithelial cells is a crucial event in alveolar liquid clearance, an event necessary to assure proper gas exchange in the alveoli, we have evaluated the capacity of the lectin-like domain of TNF, mimicked by the TIP peptide, to activate edema reabsorption in *in situ*, *ex vivo* and *in vivo* flooded rat and mouse lung models. As such, we found that the peptide was able to efficiently activate edema reabsorption, to the same extent as e.g. the beta2 adrenergic agonists, in the absence of any pro-inflammatory activity. (130-132). Interestingly, the lectin-like domain of TNF probably performs this activity in a catecholamine-independent manner (127). Moreover, whereas wt hTNF rather decreased lung liquid clearance (LLC) in a rat hydrostatic edema model *in vivo*, probably because of the TNF receptor 1-mediated effects on the expression and function of the epithelial sodium channel (133, 134), complexing the cytokine with its soluble TNF receptor 1 shifted this activity towards an activation of LLC, and *N, N'*-diacetylchitobiose could reduce this again to basal levels (132). These data could imply that physiologically, the activity of the lectin-like domain of TNF, like the dark side of the moon, could represent a function of the cytokine that is hidden by the adverse effects mediated by the TNF receptor binding sites. However, in the presence of the soluble TNF receptors, this activity can prevail, which could provide an explanation why positive effects of TNF on alveolar liquid clearance were mainly reported in infection models, which are characterized not only by increased levels of TNF, but also of its soluble receptors (135-137). In view of its profound effects on the pulmonary vasculature, the development of pulmonary edema could also occur upon treatment of cancer patients with TNF, eventually combined with Interferon-gamma and IL-2, as was reported in a phase I clinical study in patients with metastases (138), in primary or recurrent limb melanoma and sarcoma patients undergoing ILP with TNF (139) or in experimental ILP rat models combining TNF with Actinomycin D (140). Since under these conditions, substances like beta2 adrenergic agonists, which apart from anti-edema effects, also induce anti-inflammatory activities (141), would likely counteract the anti-tumor activities of TNF, the search for alternative anti-edema agents, such as the TIP peptide, remains attractive.

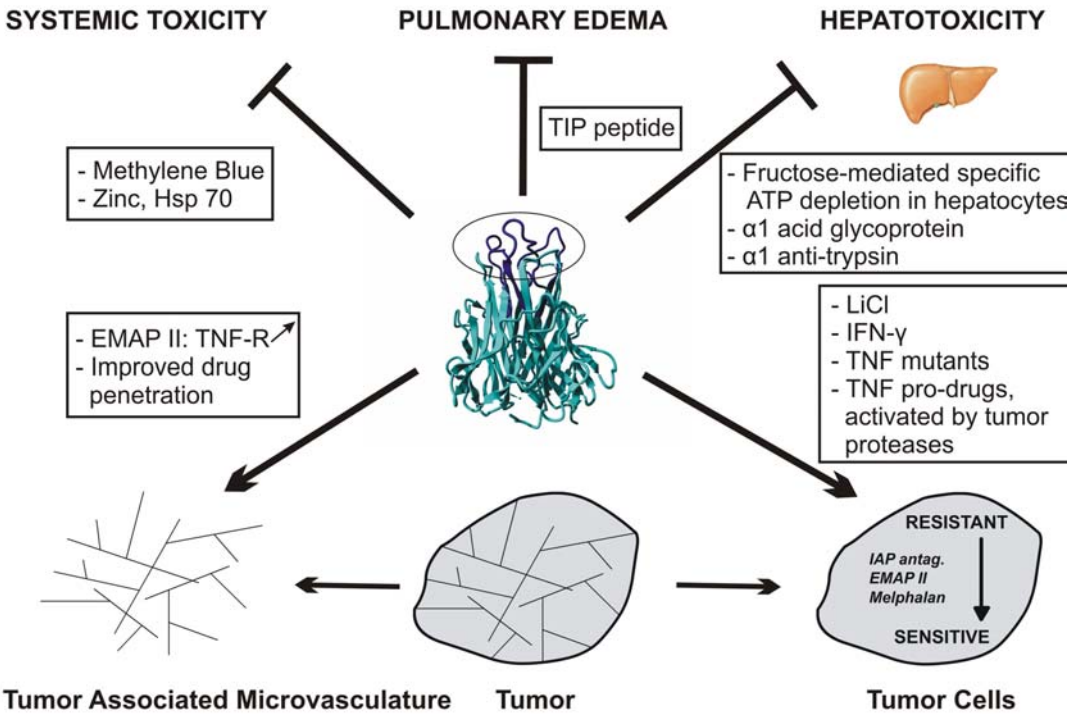

Figure 1. Overview of recent developments aiming to improve the suitability of TNF as a systemic anti-cancer agent. To this purpose the sensitivity of the tumor cells for TNF can be improved by means of blocking their anti-apoptotic defense mechanisms, using i) small IAP antagonists, which not only block IAP but also induce TNF production in the treated cells or ii) alkylating agents which block transcription, such as melphalan or iii) Lithium salts, which increase both TNF-induced apoptosis and necrosis. Also, substances such as IFN-gamma, which synergize with TNF, as well as TNF mutants with reduced systemic toxicity, but retained tumoristatic activity, as well as TNF pro-drugs that are specifically cleaved and activated at the tumor site can improve the therapeutic window. Also the effects of TNF on the tumor vasculature can be enhanced by substances such as EMAP II, which increase the expression of TNF receptors in these cells. Moreover, the profound effects of TNF on the vascular permeability can improve delivery of chemotherapeutic drugs to the tumor site. These developments must be combined with strategies that reduce the profound side effects of the cytokine, such as i) methylene Blue, hsp 70 and Zinc, which interfere with the systemic toxicity of TNF , ii) novel substances that can improve the reabsorption of pulmonary edema, which can be induced by the effects of TNF and endothelial permeability, such as the TNF-derived TIP peptide, and iii) factors inhibiting the hepatotoxicity of TNF, while preserving its tumoristatic activities, such as the acute phase proteins alpha 1 acid glycoprotein and alpha 1 anti-trypsin on the one hand, and fructose on the other hand, as the hepatocyte specific ATP depleting agent.

4. PERSPECTIVE

The initial enthusiasm about TNF as a promising anti-tumor agent has faded, in view of its prominent side effects. However, recent developments aiming at i) improving tumor cell sensitivity to TNF, as well as ii) the efficiency and specificity of TNF's apoptotic and anti-angiogenic effects on the tumor microvasculature, could renew the interest in this fascinating pleotropic cytokine as a systemic anti-cancer agent, especially when combined with strategies which can reduce these side effects, such as hypotension, hepatotoxicity and, to a lesser extent, pulmonary edema (summarized in Figure 1).

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