Early destruction of tumor vasculature in tumor necrosis factor-α-based isolated limb perfusion is responsible for tumor response

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Addition of high-dose tumor necrosis factor-α to melphalan-based isolated limb perfusion enhances anti-tumor effects impressively. Unfortunately, the mechanism of action of tumor necrosis factor-α is still not fully understood. Here, we investigated the effects of tumor necrosis factor-α on the tumor microenvironment and on secondary immunological events during and shortly after isolated limb perfusion in soft-tissue sarcoma-bearing rats. Already during isolated limb perfusion, softening of the tumor was observed. Co-administration of tumor necrosis factor-α in the isolated limb perfusion with melphalan induced a six-fold enhanced drug accumulation of melphalan in the tumor compared with isolated limb perfusion with melphalan alone. In addition, directly after perfusion with tumor necrosis factor-α plus melphalan, over a time-frame of 30 min, vascular destruction, erythrocyte extravasation and hemorrhage was detected. Interstitial fluid pressure and pH in the tumor, however, were not altered by tumor necrosis factor-α and no clear immune effects, cellular infiltration or cytokine expression were observed. Taken together, these results indicate that tumor necrosis factor-α induces rapid damage to the tumor vascular endothelial lining resulting in augmented drug accumulation. As other important parameters were not changed (e.g. interstitial fluid pressure and pH), we speculate that the tumor vascular changes, and concurrent hemorrhage and drug accumulation are the key explanations for the observed synergistic anti-tumor response. *Anti-Cancer Drugs* 17:949–959 © 2006 Lippincott Williams & Wilkins.

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Introduction

Isolated limb perfusion (ILP) with tumor necrosis factor-α (TNF-α) and melphalan, or to a lesser extent with doxorubicin, is currently one of the therapies available for the treatment of patients with advanced bulky melanoma and sarcoma of the limbs, and results in impressive enhancement of the response rates of over 80% in a great variety of tumors [1,2]. Effective tumor therapy with TNF-α requires high concentrations, a prerequisite that is limited by the toxicity of the cytokine. The development of the ILP allows loco-regional high doses with minimal risk for systemic toxicity. Investigations on the exact mechanism of the synergistic effect between TNF-α and chemotherapy in ILP are still ongoing. At the histopathological level, it was shown that 3 h after ILP the effects of TNF-α in an ILP starts with intratumoral endothelial cell activation followed by over-expression of adhesion molecules, which in turn leads to polymorphonuclear leukocyte homing, endothelium injury, and finally coagulative and hemorrhagic necrosis [3,4]. We developed a TNF-α-based ILP models in rats, with results similar to the clinical setting, to gain further insight into the mechanisms underlying the observed synergy [5,6]. In this animal model, we also observed hemorrhagic necrosis, edema, extravasation of erythrocytes, infiltration of polymorphonuclear neutrophils and destruction of tumor vasculature 24 h after perfusion [7]. The anti-tumor response is regulated by numerous factors, including cytokine production by tumor cells and other cell sources of the tumor microenvironment (endothelial cells, fibroblasts, tumor infiltrating lymphocytes), and other cytotoxic factors, such as nitric oxide. Performing an ILP with TNF-α in combination with melphalan in leukopenic rats, we found that the synergistic TNF-α effect was lost, indicating the importance of leukocytes in this model [8]. TNF-α induces the production of other cytokines [e.g. interleukin (IL)-1, IL-6 and IL-8] and cytotoxic factors (e.g. nitric oxide) by T lymphocytes,

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granulocytes and macrophages, which could mediate the tumor suppression. The complexity of the interaction of TNF-α with various immune modulators within the tumor microenvironment is not yet well defined [9–11].

These histopathological effects of TNF-α described thus far are relatively long after ILP. Early vascular changes, i.e. directly at the end of the ILP procedure, however, are unknown. In this study, we focus on the effects inflicted by TNF-α during and shortly after ILP.

Materials and methods

Animals

Male inbred BN rats, weighing 250–300 g, obtained from Harlan-CPB (Austerlitz, The Netherlands) were used for ILPs. Rats were fed a standard laboratory diet *ad libitum* (Hope Farms, Woerden, The Netherlands) and were housed under standard conditions. The experimental protocols adhered to the rules outlined in the 'Dutch Animal Experimentation Act' (1977) and the published *Guidelines of the UKCCCR for the welfare of animals in experimental neoplasia* [12]. The committee on Animal Research of the Erasmus University Rotterdam, The Netherlands, approved the protocol.

Tumor model

The rapidly growing and metastasizing BN-175 soft-tissue sarcoma, which is transplantable to the BN rat, was used. Fragments of 2–3 mm were implanted subcutaneous in the right hind limb, just above the ankle. Perfusion was performed at a tumor diameter of 13 ± 2 mm, approximately 7 days after implantation.

Drugs

Recombinant human TNF-α was provided by Boehringer Ingelheim (Vienna, Austria) with a specific activity of 5.8×10^7 U/mg as determined in the murine L-M cell assay [13]. Endotoxin levels were below 1.25 units (EU)/mg protein. TNF-α concentrations used were 50 μg in 5 ml perfusate.

Melphalan (Alkeran; Wellcome, Beckenham, UK) was diluted in phosphate-buffered saline (PBS) to a concentration of 2 mg/ml. Concentrations used were 40 μg in 5 ml perfusate.

Isolated limb perfusion model

The perfusion technique was performed as described previously [6]. Briefly, animals were anesthetized with Ketalin (Apharmo, Duiven, The Netherlands) and Xylazin (Bayer, Mijdrecht, The Netherlands). To prevent coagulation, 50 IU of heparin was injected intravenously. To keep the rat's hind limb at a constant temperature of 38–39°C, a warm water mattress was applied. Temperature was measured with a temperature probe on the skin covering the tumor. The femoral artery and vein were cannulated with silastic tubing (0.012-inch inner diameter, 0.025-inch outer diameter; 0.025-inch inner diameter, 0.047-inch outer diameter, respectively; Dow Corning, Midland, Michigan, USA). Collaterals were occluded by a groin tourniquet and isolation time started when the tourniquet was tightened. An oxygenation reservoir and a roller pump were included into the circuit. The perfusion solution was 5 ml Hemaccel (Behring Pharma, Amsterdam, The Netherlands). Melphalan with or without TNF-α was added as a bolus to the oxygenation reservoir. A roller pump (Watson Marlow, Falmouth, UK; type 505 U) recirculated the perfusate at a flow rate of 2.4 ml/min for 30 min. A washout with 5 ml oxygenated Hemaccel was performed at the end of the perfusion.

Tumor growth was recorded daily by caliper measurement. Tumor volume was calculated as $0.4 \times (A^2 \times B)$, where B represents the longest diameter and A the diameter perpendicular to B. The classification of tumor response was as follows: progressive disease = increase of tumor volume (> 25%) within 8 days; no change = tumor volume equal to initial volume (in a range of −25 and +25%); partial remission = decrease of tumor volume (−25 and −90%); complete remission = tumor volume less than 10% of initial volume. The animals with partial remission and complete remission were considered responders to the therapy.

Measurement of melphalan in tissue

At the end of the perfusion directly after the washout, the tumor and part of the hind limb muscle were excised. The tissues were immediately frozen in liquid nitrogen to stop metabolism of melphalan and stored at −80°C. Tumor and muscle tissues were homogenized in 2 ml acetonitrile (Pro 200 homogenizer; Pro Scientific, Oxford, Connecticut, USA) and centrifuged at 2500 *g*. Melphalan was measured in the supernatant by gas chromatography–mass spectrometry. *p*-[Bis(2-chloroethyl)amino]-phenylacetic methyl ester was used as an internal standard. Samples were extracted over trifunctional C_{18} silica columns. After elution with methanol and evaporation, the compounds were derived with trifluoroacetic anhydride and diazomethane in ether. The stable derivates were separated on a methyl phenyl siloxane gas chromatography capillary column and measured selectively by single-ion monitoring gas chromatography–mass spectrometry in the positive EI mode described earlier by Tjaden and De Bruijn [14].

Measurement of interstitial fluid pressure

Interstitial fluid pressure (IFP) was measured in tumor and muscle during ILP with the wick-in-needle technique [15]. A 23-gauge needle (Venisystems; Abbott Ireland, Sligo, Ireland) with a 2–3-mm side hole 5 mm

from the tip was filled with five surgical sutures 6/0 (Braun Medical, Oss, The Netherlands) and connected to a pressure transducer (DTX Plus Transducer; Becton Dickinson, Alphen aan den Rijn, The Netherlands). Pressure was recorded on an analog–digital converter (AS/3 DATEX). After cannulating the femoral artery and vein, but before applying the tourniquet, the needle was inserted in the tumor and in the muscle of the same leg. The IFP was recorded until the end of the perfusion.

pH measurements

A calibrated pH electrode in a 20-gauge needle (Harvard Apparatus, Holliston, Massachusetts, USA) was inserted in the tumor just before the roller pump was started. The pH of the perfusate was measured by inserting a portable pH meter (pH meter HI 8424; Hanna Instruments, Ann Arbor, Michigan, USA) in the oxygenation reservoir.

Hematoxylin–eosin staining

Directly or 6 h after perfusion, tumors were excised and cut in two equal parts. Both parts were divided into a peripheral part and a central part. The tissues were stored in formalin and embedded in paraffin. Sections of 4 μm were stained with hematoxylin–eosin solution (Sigma, Zwijndrecht, The Netherlands) using standard procedures. Three or four different tumors in each experimental group were subjected to blind evaluation. At least six slides were examined from each tumor. All slides were examined on a Leica DM-RXA (Leica Microsystems, Rijswijk, The Netherlands) and photographed using a Sony 3CCD DXC 950 camera (Amsterdam, The Netherlands).

TUNEL/CD31PE double staining

Apoptotic cell death was detected using the technique of 3′-hydroxy end-labeling (In Situ Cell Death Detection Kit, fluorescein labeled; Roche, Almere, The Netherlands). Tumor tissues were also stained for endothelial cells to differentiate between apoptosis of the endothelium and of tumor cells.

After ILP, the tumors were excised and immediately frozen in liquid nitrogen. Staining was performed on acetone-fixed 7-μm cryostat sections. The tumor sections were fixed in 4% paraformaldehyde for 30 min and incubated for 1 h with 1:50 mouse anti-rat CD31–phycoerythrin (Becton Dickinson). After washing with PBS the sections were again fixed in 4% paraformaldehyde for 10 min and incubated in 0.1% Triton X-100 in 0.1% sodium citrate for 2 min on ice to allow permeabilization. The slides were incubated with the TUNEL mixture for 1 h at 37°C, and after that the slides were rinsed three times in PBS and mounted with mounting medium containing polyvinyl alcohol (Mowiol 4-88; Fluka, Zwijndrecht, The Netherlands). Endothelial cells were identified by red fluorescence and apoptosis by green fluorescence. Apoptotic endothelial cells were detected by co-localization of red and green (displayed as yellow) fluorescence.

Immunohistochemistry

Directly or 6 h after ILP, tumors were excised and frozen in liquid nitrogen. Immunohistochemical staining was performed on acetone-fixed 7-μm cryostat sections. The tumor sections were fixed for 30 min with 4% formaldehyde and after rinsing with PBS the endogenous peroxidase activity was blocked by incubation for 5 min in methanol/3% H_2O_2. The slides were incubated for 1 h with 1:50 mouse anti-rat-CD31, -CD4, -CD8, -granulocytes (clone HIS48; Becton Dickinson) and ED-1 (Serotec, Breda, The Netherlands). Thereafter, sections were washed with PBS and incubated for 1 h with 1:100 goat anti-mouse peroxidase-labeled antibody (ITK Diagnostics Uithoorn, The Netherlands). After rinsing with PBS, positive cells were revealed by immunoperoxidase reaction with DAB solution (ITK Diagnostics) and counterstained lightly with hematoxylin (Sigma).

For quantification of infiltration and microvessel density, two independent persons performed blinded analysis. Six representative fields (magnification × 16) in each slide and three tumors per treatment were evaluated. The sections were examined on a Leica DM-RXA and photographed using a Sony DXC950 camera. For T cell, granulocyte and macrophage infiltration, the total amount of positive cells was counted per field of interest. For the microvessel quantification, the number of tumor blood vessels and the area of vessels per field of interest were measured in calibrated digital images (Research Assistant 3.0; RVC, Hilversum, The Netherlands). The average size of a vessel was calculated by dividing the number of vessels with the area of vessels per field of interest.

Semi-quantitative reverse-transcriptase polymerase chain reaction

Total RNA was extracted from frozen tumor tissue using TRIzol reagent as suggested by the manufacturer (Invitrogen, Breda, The Netherlands). cDNA synthesis was carried out by incubating 1.0 μg RNA with Omniscript Reverse Transcriptase (Qiagen, Leusden, The Netherlands) and oligo $d(T)_{16}$ (Invitrogen) for 1 h at 42°C after heating for 5 min at 93°C to terminate the reaction.

Polymerase chain reaction (PCR) reaction was performed with a Biometra T-gradient PCR machine on a reaction mix of 1.5 μl cDNA, 200 nmol/l deoxynucleoside triphosphate, 1.5 mmol/l $MgCl_2$, 50 pmol/l of the sense and antisense primers, and 0.5 U titanium Taq DNA polymerase (Becton Dickinson) in PCR buffer to 50 μl final volume.

The primers were purchased from Invitrogen and the primer sequences are shown in Table 1. β-Actin was used as an internal standard. For PCR we used the following parameters: initial denaturation at 94°C for 5 min followed by a maximum of 40 cycles of 94°C for 45 s, annealing for 45 s (temperatures: see Table 1) and extension 72°C for 1 min. To obtain results from the exponential phase of the reaction every two cycles, 5 µl of PCR product was collected, and the samples were separated on a 1.5% agarose gel, stained with ethidium bromide and photographed under ultraviolet light. A 100-bp ladder was used as the standard. The threshold cycle was determined as the cycle in which the visible band of a specific PCR product first appeared on the gel. The results were expressed as the cycle of first appearance of the band normalized with the cycle of first appearance of β-actin.

Statistical analysis

Results were evaluated for statistical significance with the Mann–Whitney *U* test. *P* values below 0.05 were considered statistically significant. Calculations were performed on a personal computer using GraphPad Prism 3.0 (GraphPad Software, San Diego, California, USA) and SPSS 11.0 for Windows 2000 (SPSS, Chicago, Illinois, USA).

Results

In-vivo response to melphalan and tumor necrosis factor-α

Combination of TNF-α and melphalan resulted in an increased anti-tumor activity with a response rate (partial and complete remission) of 80% compared with perfusion with melphalan alone ($P < 0.001$). Progressive disease was found in all animals treated with sham or TNF-α alone. Perfusion with melphalan alone showed some inhibition of tumor growth compared with sham and TNF-α, resulting in 17% response. This tumor response increased to 80% when TNF-α was added (Table 2). Another important observation made was the softening of the tumor, during the 30 min of perfusion, when treated with TNF-α in combination with melphalan, a characteristic we did not observed in the other treatments.

Melphalan uptake in tumor and muscle tissue

Immediately after perfusion with melphalan or melphalan in combination with TNF-α tumor and muscle were excised, and melphalan concentration was measured. In tumor tissue perfused with melphalan and TNF-α, we measured a six-fold increased melphalan uptake compared with tumor tissue treated with melphalan alone ($P = 0.01$). That an increased uptake of melphalan by TNF-α is specific for the tumor is demonstrated by the fact that in skin and muscle no effect of TNF-α was found on the uptake of melphalan (Table 2). The melphalan concentration increased from 136 ng/g tissue to 831 ng/g tissue when TNF-α was co-administered. The IC_{50} (concentration of a drug that is required for 50% inhibition of cell growth) of melphalan on BN175 cells is 250 ng/ml [16], demonstrating that the addition of TNF-α to the ILP shifts the intratumoral drug concentration to a cytotoxic range. Addition of TNF-α does not change the sensitivity of BN175 cells *in vitro*, indicating that only melphalan has a cytotoxic effect on the tumor cells. This indicates that the increased intratumoral melphalan concentration is responsible for the cytotoxic effect on the tumor cells leading to tumor regression. It is important to mention that the IC_{50} of melphalan on

Table 1 Reverse-transcriptase polymerase chain reaction primers for the immune-related genes

Gene	Primers	Annealing temperatures (°C)	Product size (bp)
β-Actin	f: 5′-ATGGATGACGATATCGCTG-3′ r: 5′-ATGAGGTAGTCTGTCAGGT-3′	60	569
IL-6	f: 5′-GACTTCACAGAGGATACC-3′ r: 5′-TAAGTTGTTCTTCACAAACTCC-3′	55	294
GRO/CINC-A	f: 5′-GAAGATAGATTGCACCGATG-3′ r: 5′-CATAGCCTCTCACACATTTC-3′	57	367
IL-10	f: 5′-TGACAATAACTGCACCCACTT-3′ r: 5′-TCATTCATGGCCTTGTAGACA-3′	60	402
IL-12	f: 5′-TCATCAGGGACATCATCAAACC-3′ r: 5′-CGAGGAACGCACCTTTCTG-3′	65	210
TNF-α	f: 5′-TACTGAACTTCGGGGTGATCGGTCC-3′ r: 5′-CAGCCTTGTCCCTTGAAGAGAACC-3′	60	295
IFN-γ	f: 5′-GCCTCCTCTTGGATATCTGG-3′ r: 5′-GTGCTGGATCTGTGGGTTG-3′	60	239
MCP-1	f: 5′-ATGCAGGTCTCTCTGTCACG-3′ r: 5′-CTAGTTCTCTGTCATACT-3′	57	446
MIP-2	f: 5′-GGCACAATCGGTACGATCCAG-3′ r: 5′-ACCCTGCCAAGGGTTGACTTC-3′	55	287
TGF-β1	f: 5′-TGGAAGTGGATCCACGAGCCCAAG-3′ r: 5′-GCAGGAGCGCACGATCATGTTGGAC-3′	55	240

β-Actin was used as a housekeeping gene.
f, forward primer; r, reverse primer; IL, interleukin; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ; MCP-1, monocyte chemoattractant protein-1; MIP-2, macrophage inflammatory protein-2; TGF-β1, transforming growth factor-β1; GRO/CINC-A, growth regulated gene product/cytokine-induced neutrophil chemoattractant (rat analog for IC-8).

Table 2 Tumor response and drug accumulation after TNF-α-based ILP with melphalan

Treatment	Tumor volume at day 8 (mm^3)[a]	Percentage response rate[b]				Melphalan (ng/g tissue)[c]	Melphalan in skin/muscle (ng/g tissue)[c]
		PD	NC	PR	CR		
Sham	>5000	100					
TNF-α	4570 ± 511	100					
Melphalan	1918 ± 293	42	33	17		136 ± 24	316 ± 91
TNF-α + melphalan	491 ± 245	13	7	40	40*	831 ± 293**	290 ± 125

TNF-α, tumor necrosis factor-α; ILP, isolated limb perfusion; PD, progressive disease; NC, no change; PR, partial remission; CR, complete remission. *P<0.001 compared with melphalan alone. **P=0.001 compared with melphalan alone.
[a]Average ± SEM.
[b]Responses were scored as described in Materials and methods.
[c]Average ± SD.

endothelial cells is much higher (40 000 ng/ml), which implies that endothelial cells are most likely not affected by melphalan in the ILP setting [17].

Histological analysis

Previously, we demonstrated vascular damage, extravasation of erythrocytes and necrosis, causing drastic alterations in permeability and integrity of the vasculature 24 h after ILP with melphalan in combination with TNF-α [7]. In hematoxylin–eosin staining directly after perfusion with TNF-α plus melphalan, an increase in the number and size of necrotic areas was observed accompanied by scattered extravasation of erythrocytes (Fig. 1). In some areas, we detected destruction of the vasculature and from these vessels infiltration of erythrocytes into the surrounding tumor tissue occurred. In tumors treated with TNF-α alone, we also observed destruction of the vasculature and erythrocyte infiltration from these vessels. After melphalan or sham treatment, no extravasation of erythrocytes, necrotic areas or loss in endothelial integrity was observed. Strikingly, no clear differences between the central and peripheral parts of the tumors were seen directly or 6 h after ILP, with the exception that after 6 h, slightly more necrotic areas were detected (data not shown).

For the detection of apoptotic endothelial cells, we performed a TUNEL/CD31 double staining (Fig. 2). In all four treatments, apoptotic tumor cells were observed sporadically. In the TNF-α and TNF-α plus melphalan-treated tumors an increased number of apoptotic endothelial cells was detected directly after perfusion.

Interstitial fluid pressure measurements in tumor and muscle tissue

As TNF-α was shown to reduce IFP in tumor tissue in a systemic treatment [18], IFP measurements were performed in both tumor and muscle tissue during ILP to investigate whether TNF-α-induced lowering of the IFP could be an explanation for the enhanced drug accumulation and softening of the tumor.

Under normal conditions, the IFP of tumor tissue is much higher than that of muscle tissue (Fig. 3a). Tightening of the tourniquet resulted in an increased IFP in both tissues and start of the perfusion pump did not further increase the IFP. None of the treatments had an effect on the IFP in the tumor, and the only increase we could see was in the muscle of the melphalan-treated group after 20 and 30 min ($P = 0.02$). We speculated that the pressure inflicted by the pump in the extracorporal circuit might have a major impact on the IFP. Indeed, when we increased the pump rate in our animal model, tumor IFP increased and decreased again when the pump rate was reduced (data not shown). Although only one test was performed, clearly the effect of the pump rate on the IFP was confirmed in the clinical setting in which comparable results were obtained (Fig. 3b).

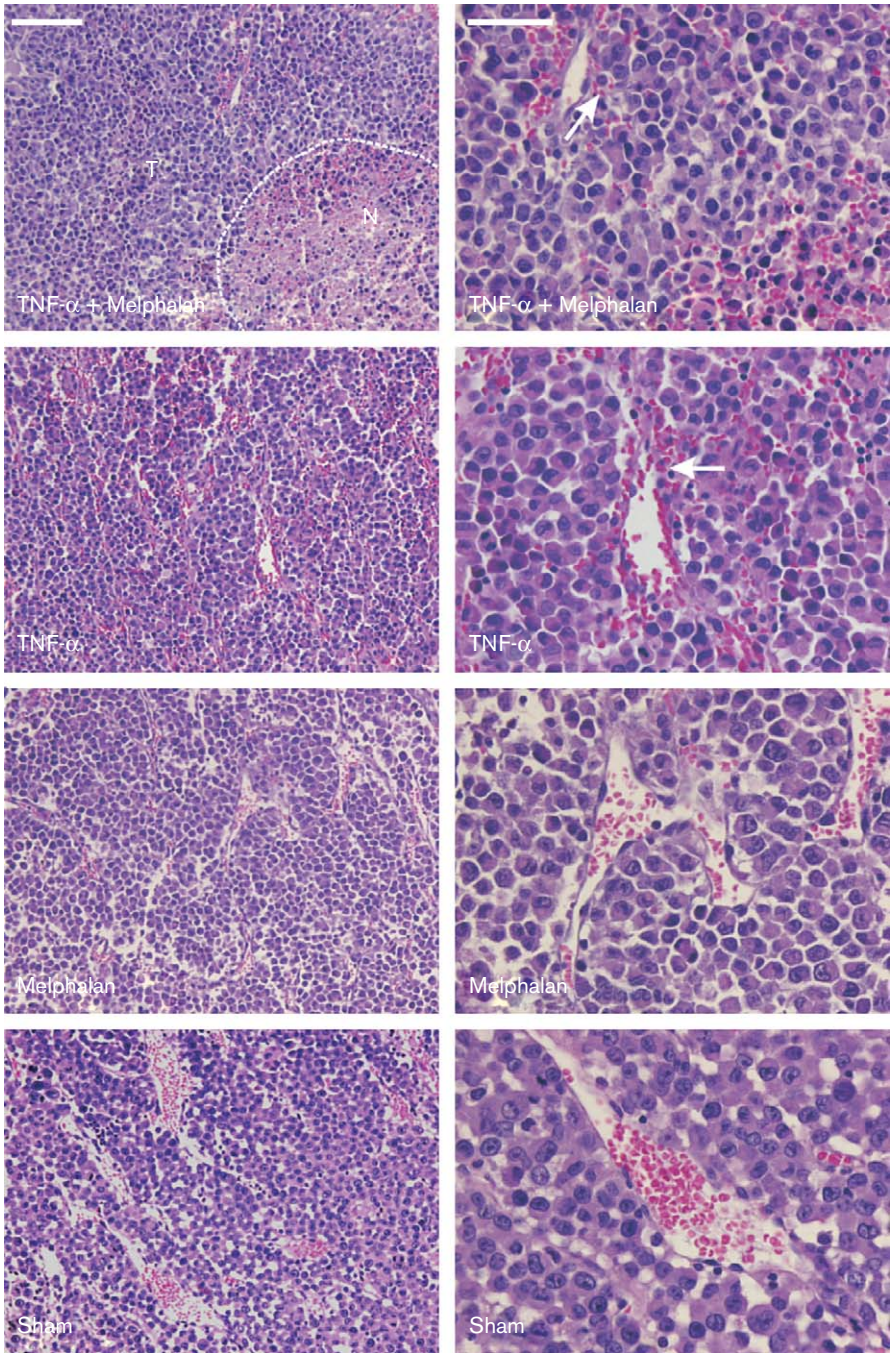
pH measurements in tumor and perfusate

Tumor acidity can increase the anti-tumor activities of weak acid chemotherapeutics like melphalan [19]. Although acidosis is already a known fact in the microenvironment of solid tumors and is found in hypoxic areas, we tested the hypothesis whether treatment with TNF-α could cause a lowering of pH in tumor tissue. The pH of the tumor at the beginning of the perfusion was slightly acidic (6.9 ± 0.1) and lowered during perfusion until 6.6 ± 0.3. The pH of the perfusate was 6.9. Adding the drugs had no effect, whereas oxygenation lowered the pH to 6.2. Directly after start of the perfusion the pH of the perfusate increased to 6.5 ± 0.3 and was 6.9 ± 0.1 at the end of perfusion. None of the treatments had an effect on the pH of the tumor or the perfusate indicating that TNF-α does not induce acidosis in this setting.

Assessment of tumor vascular functionality

The increased uptake of melphalan might be correlated with the functionality of the tumor-associated vasculature. Quantification of the microvessel density and functionality was performed by immunohistochemical staining of endothelial cells. The number of vessels and vessel area was measured. The area per vessel was computed by dividing the total area of vessels by the number of vessels. Although we observed endothelial damage in the hematoxylin–eosin staining, addition of

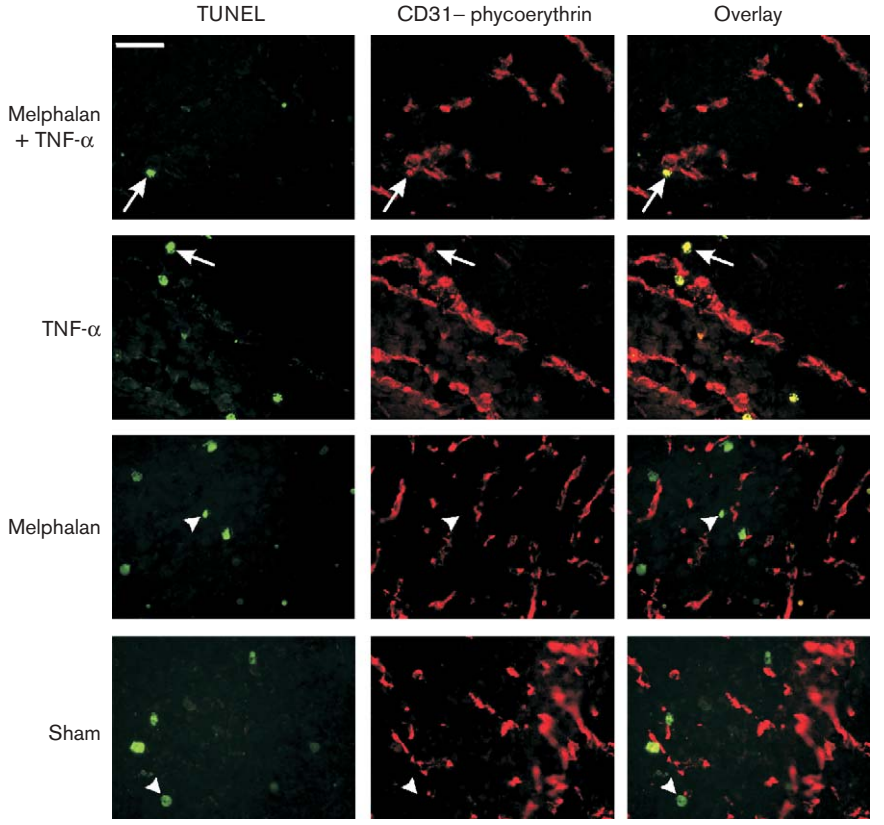
Fig. 1



Effect of tumor necrosis factor-α (TNF-α) on the histopathology of tumor tissue after isolated limb perfusion. Hematoxylin–eosin staining directly after perfusion with melphalan in combination with TNF-α showed necrotic areas, destruction of the vascular lining and extravasation of erythrocytes. Perfusion with TNF-α alone also induces vascular damage and erythrocyte infiltration. In sham or melphalan-treated tumors the vessel lining is still intact. Arrow: extravasated erythrocytes, dotted line denotes the boundary between normal tumor tissue (T) and necrotic tissue (N). Original magnification × 16, scale bar: 100 μm (left panel) and × 40, scale bar: 50 μm (right panel).

TNF-α had no effect on the number and size of the vessels directly after perfusion (Table 3). The vessel area in the center or peripheral part of the tumor was significantly decreased 6 h after ILP compared with the area measured directly after perfusion with sham, TNF-α or melphalan ($P = 0.05$); however, no change was observed after perfusion with TNF-α in combination with melphalan.

Fig. 2



Effect of tumor necrosis factor-α (TNF-α) on apoptosis of endothelial cells in tumor directly after isolated limb perfusion. Photographs demonstrating apoptotic tumor cells (green), endothelial cells (red) and apoptotic endothelial cells (yellow). Perfusion with melphalan in combination with TNF-α or with TNF-α alone induces apoptosis of endothelial cells, whereas in the staining of tumor tissue after sham or melphalan perfusion no apoptotic endothelial cells were observed. Arrow: apoptotic endothelial cells, arrowhead: apoptotic tumor cell. Original magnification × 40, scale bar: 50 μm.

Secondary immunological effect of tumor necrosis factor-α

Tumor infiltration of leukocytes and macrophages

In a previous study of ILP with IL-2 in combination with melphalan, we observed a redistribution of macrophages that was not observed in a sham perfusion [20]. In-vitro data showed that TNF-α has only an effect on endothelial cells when combined with interferon-γ (IFN-γ) and peripheral blood mononuclear cells [17]. Therefore, we also examined the possible secondary effects of TNF-α in the ILP. Performing ILP with TNF-α, directly or 6 h after perfusion, we found no evident alterations in number (Table 4) of localization (Fig. 4) of the examined tumor infiltrating cells in all four treatments groups.

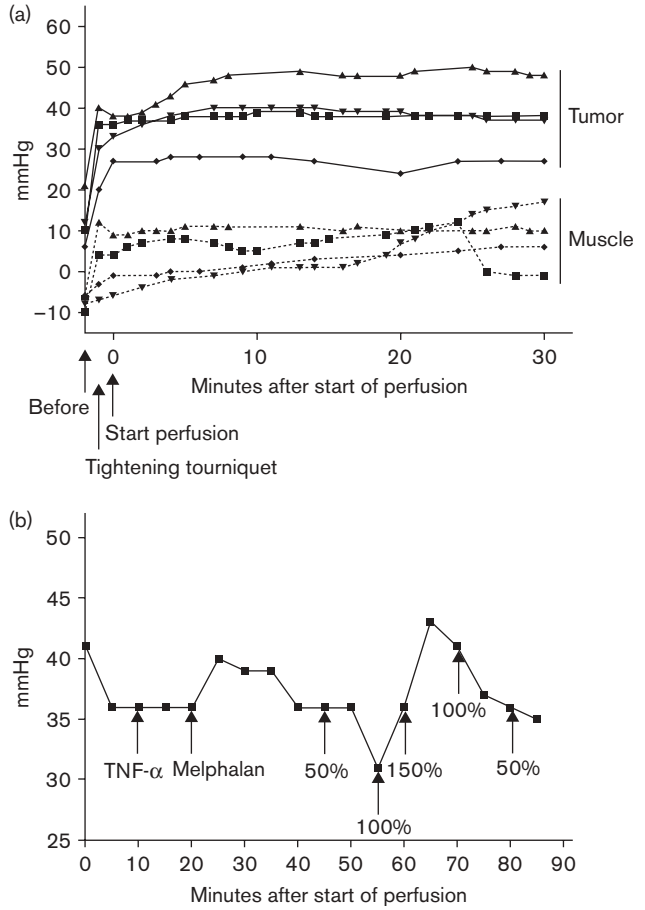
Cytokine expression in tumor tissue

As the number of tumor-infiltrating cells was not affected by TNF-α, we hypothesized that TNF-α could probably activate macrophages and T lymphocytes resulting in the production of non-specific effector molecules. From the nine cytokines examined, however, we found no change in expression profile between the treatments groups (Table 5).

Discussion

Clinical studies showed impressive improvement of the anti-tumor activity of melphalan in local treatment of different tumor types when TNF-α was co-administered [1,2]. In this paper and in previous studies, we demonstrate that ILP with melphalan in combination with TNF-α in soft-tissue sarcoma-bearing rats results in high response rates. This combination therapy is also very effective for the rat osteosarcoma and with another chemotherapeutical agent, i.e. doxorubicin [21,22]. We demonstrated that the basis for the synergy is a significant enhancement of tumor drug accumulation. Subsequent events were selective destruction of the tumor-associated vasculature leaving the normal vessel of the targeted area intact. This impaired blood flow leads to edema and necrosis. Although we observed these features already in rodent tumor models with TNF-α alone, no significant tumor regression occurred in these animals. Further, these effects were observed at least 24 h after perfusion, whereas the tumor-specific uptake of the chemotherapeutic drug occurred within 30 min of perfusion. Melphalan is an alkylating agent and has a cytotoxic effect on dividing tumor cells, whereas TNF-α works

Fig. 3



Effect of tumor necrosis factor-α (TNF-α) on interstitial fluid pressure (IFP) during isolated limb perfusion (ILP). During perfusion with sham (■), TNF-α (▲), melphalan (▼) or melphalan in combination with TNF-α (◆) the IFP was continuously recorded as described in Materials and methods in tumor (continuous line) and muscle (dotted line). At least three animals per group were measured. No differences in the effect of TNF-α on the IFP were observed between the four treatments (a). Decreasing perfusion pump rate during ILP in a patient resulted in a lower IFP and vice versa, indicating that in an ILP the IFP is dictated by the pump pressure (b).

Table 3 Microvessel density and area of the tumor vessels after isolated perfusion with sham, TNF-α, melphalan or TNF-α plus melphalan

	Sham	TNF-α	Melphalan	TNF-α + melphalan
Number of vessels[a]				
Center 0 h	19 ± 6	21 ± 7	22 ± 2	34 ± 25
Periphery 0 h	43 ± 12	32 ± 9	34 ± 6	19 ± 5
Center 6 h	46 ± 31	34 ± 13	32 ± 14	18 ± 7
Periphery 6 h	36 ± 15	29 ± 5	38 ± 26	25 ± 6
Total vessel area[b]				
Center 0 h	7.6 ± 0.7	10.7 ± 2.7	5.9 ± 0.9	5.7 ± 1.0
Periphery 0 h	7.6 ± 0.3	7.2 ± 0.4	7.2 ± 0.1	5.0 ± 1.1
Center 6 h	4.0 ± 1.1*	4.0 ± 0.7*	6.1 ± 1.4	7.2 ± 2.4
Periphery 6 h	3.8 ± 0.5*	3.5 ± 0.4*	3.9 ± 1.5*	3.6 ± 0.5
Vessel size[c]				
Center 0 h	0.44 ± 0.09	0.59 ± 0.25	0.27 ± 0.03	0.41 ± 0.17
Periphery 0 h	0.22 ± 0.08	0.28 ± 0.10	0.23 ± 0.03	0.30 ± 0.08
Center 6 h	0.16 ± 0.05*	0.14 ± 0.0*	0.22 ± 0.03	0.48 ± 0.16
Periphery 6 h	0.14 ± 0.04	0.13 ± 0.02	0.18 ± 0.08	0.18 ± 0.07

Directly or 6 h after isolated limb perfusion, the tumors were excised and sections of the center or periphery were stained for CD31-positive cells. Six fields of interest per tumor and three animals per group were quantified. Average ± SEM is shown. *P = 0.05 compared with 0 h and the same tumor region. TNF-α, tumor necrosis factor-α.
[a]Number of vessels per field of interest.
[b]Percentage of total vessel area per field of interest.
[c]Percentage size of one vessel per field of interest.

Table 4 Tumor infiltration after isolated perfusion with sham, TNF-α, melphalan or TNF-α plus melphalan

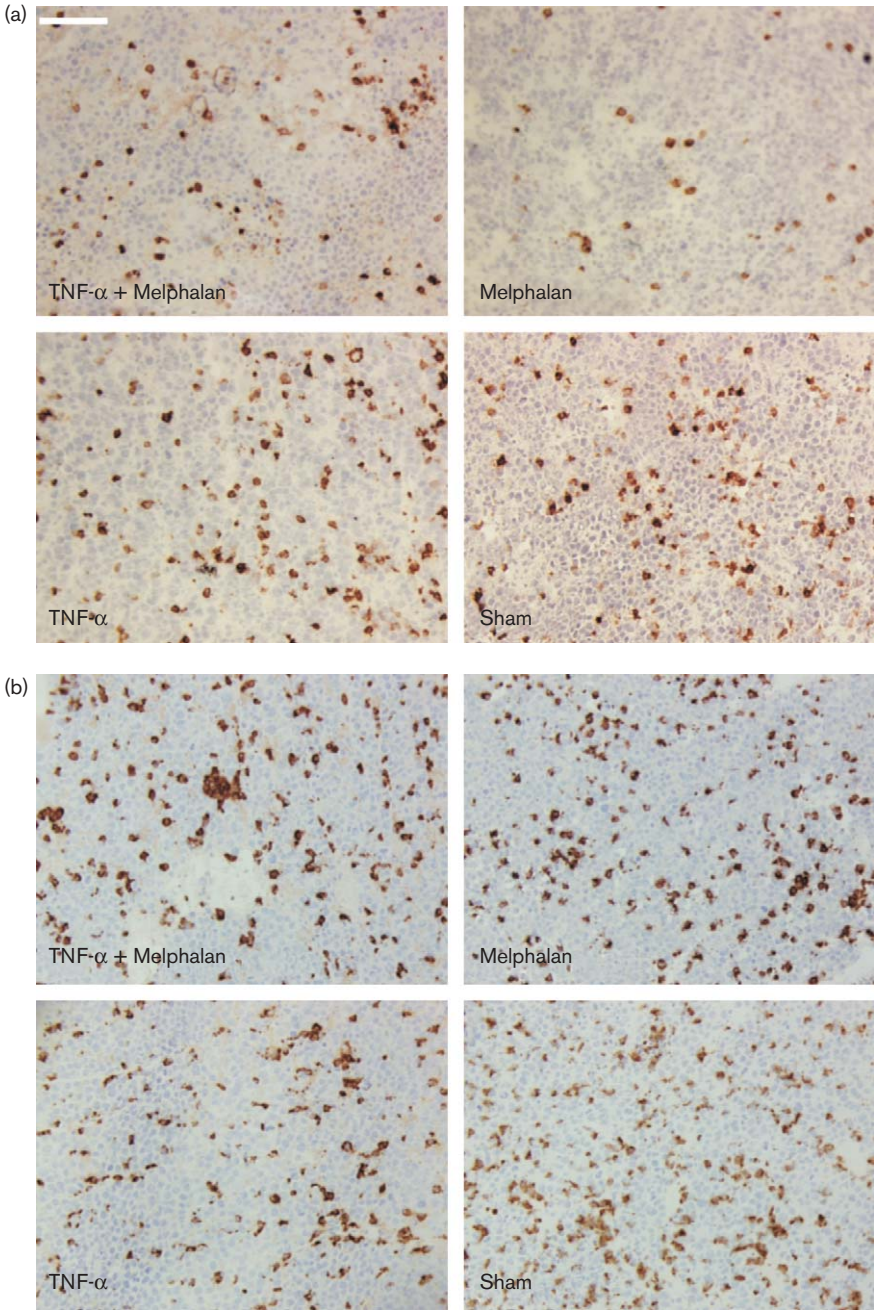
	Sham	TNF-α	Melphalan	TNF-α + melphalan
CD4[a]				
Center 0 h	0.3 ± 0.1	0.5 ± 0.3	0.2 ± 0.1	13.5 ± 13.0
Periphery 0 h	1.0 ± 0.5	1.4 ± 0.4	0.7 ± 0.5	0.5 ± 0.3
Center 6 h	1.0 ± 0.5	0.9 ± 0.4	1.2 ± 0.4	0.4 ± 0.1
Periphery 6 h	0.5 ± 0.3	0.6 ± 0.2	0.8 ± 0.3	1.6 ± 0.4
CD8[a]				
Center 0 h	34 ± 21	27 ± 21	67 ± 47	56 ± 55
Periphery 0 h	64 ± 26	35 ± 7	75 ± 27	139 ± 25
Center 6 h	22 ± 12	51 ± 16	41 ± 13	48 ± 29
Periphery 6 h	62 ± 20	33 ± 3*	51 ± 7	42 ± 8[#]
Granulocytes[a]				
Center 0 h	60 ± 4	89 ± 18	48 ± 3*	88 ± 12
Periphery 0 h	74 ± 19	81 ± 25	62 ± 10	37 ± 5
Center 6 h	69 ± 10	72 ± 18	79 ± 14	47 ± 6*,[#]
Periphery 6 h	109 ± 39	71 ± 12	50 ± 13	81 ± 12[#]
Macrophages[a]				
Center 0 h	189 ± 5	192 ± 33	201 ± 20	172 ± 34
Periphery 0 h	221 ± 42	165 ± 11	207 ± 23	239 ± 11
Center 6 h	171 ± 34	174 ± 18	230 ± 29	147 ± 10
Periphery 6 h	246 ± 39	214 ± 34	180 ± 16	189 ± 2[#]

Directly or 6 h after isolated limb perfusion, the tumors were excised and sections of the center or periphery were stained for CD4, CD8, granulocytes and macrophages. Six fields of interest per tumor and three animals per group were quantified. Average ± SEM is shown. *P = 0.05 compared with sham treatment at same time point and region of the tumor. [#]P = 0.05 compared with 0 h and the same tumor region. TNF-α, tumor necrosis factor-α.
[a]Number of positive cells per field of interest.

selectively on tumor-associated vasculature. We therefore hypothesize that ILP with melphalan and TNF-α follows a two-way mechanism. First, TNF-α targets the tumor vasculature, therefore facilitating access of melphalan in the tumor tissue followed by destruction of the tumor cells by melphalan.

Here, we focus on the direct effects of TNF-α on the tumor microenvironment and secondary immunological effects during and shortly after perfusion. Already during perfusion with TNF-α and melphalan softening of the tumor was observed, a feature also observed in the clinical setting [23], which could be an indication of hemorrhage or inflammation. Histological analysis of these tumors revealed some areas with complete destruction of the vasculature. The size and number of tumor vessels were not affected by the treatments this shortly after perfusion. We hypothesize that the slightly increased apoptotic cell number found directly after ILP only partly explain the vascular damage caused by TNF-α in combination with melphalan. Ruegg *et al.* [24] showed that inhibition of the $\alpha_V\beta_3$-mediated endothelial cell adhesion results in apoptosis, and finally disruption of the

Fig. 4



Effect of tumor necrosis factor-α (TNF-α) on number and redistibution of granuloctes and macrophages in tumor tissue directly after isolated limb perfusion. No change was observed in the number or redistribution of the tumor-infiltrating granulocytes (a) or macrophages (b) between the treatments. Original magnification × 16, scale bar: 100 μm.

tumor vasculature induced by TNF-α and IFN-γ; however, in this study tumor biopsies were examined 24 h after ILP, which might therefore be a secondary effect.

After perfusion with TNF-α in combination with melphalan or with TNF-α alone, we observed scattered extravasation of erythrocytes in the surrounding tissue. This is not seen in sham or melphalan-treated tumors. It is reported that erythrocytes can play an important role in the transport of certain drugs [25,26]. Little, however, is known about the contribution of erythrocytes in the transport of melphalan. Wildiers *et al.* [27] showed that 1 h after intraperitoneal administration of melphalan 25%

Table 5 Cytokine expression after isolated perfusion with sham, TNF-α, melphalan or TNF-α plus melphalan

	Sham[a]		TNF-α[a]		Melphalan[a]		TNF-α + melphalan[a]	
	0 h	6 h	0 h	6 h	0 h	6 h	0 h	6 h
IL-6	10 ± 1	8 ± 2	9 ± 0	6 ± 2	9 ± 0	9 ± 0	10 ± 1	7 ± 2
GRO/CINC-A	10 ± 1	5 ± 2*	7 ± 1	5 ± 3	10 ± 0	5 ± 1*	10 ± 2	4 ± 2*
IL-10	13 ± 1	13 ± 1	15 ± 4	13 ± 1	15 ± 1	13 ± 1	15 ± 1	12 ± 2
IL-12	15 ± 1	17 ± 3	14 ± 0	15 ± 3	13 ± 1	17 ± 1*	14 ± 0	16 ± 2
TNF-α	19 ± 1	13 ± 1*	17 ± 1	13 ± 2*	18 ± 2	14 ± 0*	17 ± 1	13 ± 1*
IFN	15 ± 1	11 ± 2	15 ± 1	13 ± 2	15 ± 1	13 ± 1	14 ± 0	13 ± 1
MCP-1	6 ± 1	5 ± 2	6 ± 1	6 ± 2	5 ± 0	6 ± 1	7 ± 0	6 ± 1
MIP-2	11 ± 1	8 ± 3	10 ± 3	7 ± 2	12 ± 1	9 ± 0*	12 ± 1	9 ± 2
TGF	3 ± 1	6 ± 2	4 ± 0	4 ± 2	3 ± 1	6 ± 0*	3 ± 1	5 ± 1

Directly or 6 h after isolated limb perfusion, the tumors were excised and semiquantitative reverse-transcriptase polymerase chain reaction performed. Three tumors per group were quantified. Average ± SD is shown. *$P < 0.05$ compared with 0 h. TNF-α, tumor necrosis factor-α; IL, interleukin; IFN, interferon; TGF, transforming growth factor; GRO/CINC-A, growth regulated gene product/cytokine-induced neutrophil chemoattractant (rat analog for IL-8).
[a]Numbers given are the cycles in which the first band of each cytokine appeared corrected for the first band of β-actin of the same sample.

of the melphalan in the blood was transported via erythrocytes. He also showed that although the transport of melphalan via erythrocytes was less than that in plasma, it correlated well with melphalan availability in embryonic stem cell-derived tumors [27]. An increased extravasation of melphalan-encapsulated erythrocytes by TNF-α could be a plausible explanation for the augmented concentration of melphalan in the tumor environment and further experiments on the role of erythrocytes are currently being performed in our laboratory.

Tumors are characterized by dilated hyperpermeable and irregular-shaped blood vessels, a lack of functional lymphatics, and an abnormal turnover of extracellular matrix components. Fluid and serum proteins leak from the vessels and accumulate in the interstitium, increasing the IFP. This chaotic vascular structure and diminished buffering capacity of the IFP contributes to an acidic microenvironment of the tumor. Poor uptake of drugs into tumor interstitium is thought to, at least in part, be responsible for the low efficiency in pharmacological treatment of solid malignancies [28,29]. Kristensen *et al.* [18] showed that TNF-α caused a reduction in the IFP in human melanoma xenografts, which could lead to enhanced uptake of large molecules. It has been demonstrated that lowering pH condition potentates the melphalan toxicity towards several tumor cell lines [19,30]. In our experiments, we did not see an effect of TNF-α on the IFP during perfusion. Moreover, the pressure measured was solely ascribed to the design of this model. In our study, we found that the pH in the BN175 tumor is slightly acidic and none of the treatments had an effect on the pH in the tumor during ILP.

TNF-α is able to up-regulate expression of several cytokines (IL-1, IL-6, IL-8, IFN-γ) in cancer and inflammatory responses. Up-regulation of these cytokines in the tumor microenvironment can count as a secondary effect of TNF-α. Tumor-infiltrating lymphocytes that express these cytokines are equally important. In-vitro studies showed that TNF-α alone has hardly a direct effect on endothelial cells, only in combination with IFN-γ and peripheral blood mononuclear cells did we observe morphological changes of the endothelial layer leading to increased permeability [17]. Mocellin *et al.* [31] found an increase in CD4 mRNA expression in the tumor microenvironment 24 h after ILP with TNF-α and doxorubicin compared with tumor tissue before treatment. An increased TIA-1 gene expression in tumor biopsies 24 h after TNF-α-based ILP compared with the patients treated with doxorubicin alone was found. They observed no change in tumor-infiltrating lymphocytes and some other relevant cytokines, like IFN-γ, IL-10 and transforming growth factor-β. After in-vitro stimulation with TNF-α of the different cell types that represent the tumor environment, TIA-1 expression is significantly increased in endothelial cells and NK cells. These experiments indicate that TNF-α-induced TIA-1 over-expression might sensitize endothelial cells to pro-apoptotic stimuli present in the tumor microenvironment and enhance NK cell cytotoxic activity against cancer cells [32]. Directly or 6 h after ILP, we also found no effect on the expression profile of the panel of cytokines we tested and observed no variations in number or re-localization of tumor-infiltrating lymphocytes between the treatments. A limitation of using the complete tumor for gene profiling is that we cannot link gene expression with a specific subset of cells. Further experiments are currently being performed to investigate the gene expression in the different cell sources of the tumor and the importance of a local up-regulation or down-regulation of cytokines. The imbalance in the cytokine profile within the tumor microenvironment more than the absolute level of an individual cytokine may be responsible for an effective versus ineffective immune response and needs to be further exploited.

In conclusion, application of TNF-α in combination with melphalan in an ILP strongly improves response rates,

which is due to an augmented melphalan accumulation in the tumor during ILP. We have recently reported a similar and crucial activity of histamine and IL-2 in this setting [16,20]. This increased drug accumulation by TNF-α is not explained by a lowering of the IFP. The tumor endothelial lining, however, is damaged in TNF-α-based ILP, rendering the vessels locally more permeable not only to small molecules, but also to erythrocytes that could function as carriers for melphalan. The exact mechanism of TNF-α-induced damage to the endothelial lining could not be explained by changes in cytokine expression or a redistribution of infiltrating lymphocytes around the vessels as we observed after IL-2-based ILP, although a local imbalance of cytokines around these vessels could contribute to permeability changes and will be further investigated.

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