# Cancer, Cytokines and the Acute Phase Response

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Kanker, cytokines en de acute fase reactie

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# CHAPTER 1

## INTRODUCTION AND AIMS OF THE THESIS

## 1.1 General introduction

In this chapter we summarize the present knowledge about the cytokine tumor necrosis factor-alpha (TNFα). This precedes the first chapters of this thesis, which deal with the immunomodulatory effect of exposure to a single dose of TNFα by an isolated limb perfusion in patients with extremity tumors (chapter 2-4), or to repeated doses of TNFα in patients with mesothelioma (chapter 5). In the second part of this chapter the influence of cytokines on the ongoing acute phase response is described. This is an introduction to chapter 4-6. Finally the aims of this thesis are formulated.

## 1.2 Tumor necrosis factor-alpha

### *1.2.1 History of TNFα*

The history of tumor necrosis factor-alpha (TNFα) started in 1893, the year that William Coley observed that patients with streptococcal infections sometimes developed a partial remission of some malignancies.[1] In 1944 bacterial lipopolysaccharide (LPS) was demonstrated as the active factor that induced hemorrhagic necrosis of transplantable tumors in mice.[2] The same group showed in 1962 that a serum factor induced in mice injected with LPS was responsible for hemorrhagic necrosis of tumors.[3] This factor was named tumor necrosis factor by Carswell et al. in 1975.[4] They characterized the induced factor in the serum of bacillus Calmette Guerin (BCG)- infected mice treated with endotoxin.
Because of high expectations to TNF as an antitumor agent great effort in many laboratories led to its purification, cloning and expression.[5-9]
The availability of pure recombinant TNF in large quantities allowed detailed examinations of its biological properties. TNF was found to be involved in many biological processes, e.g. host defense against infection, cachexia[10,11], septic shock and its anti-tumor effect was confirmed.

### *1.2.2 Characteristics of TNFα*

TNF is predominantly produced by activated macrophages/monocytes[12-14] and to a less extent by other cell types such as lymphocytes[15], fibroblasts and epithelial cells[16] induced by a variety of stimuli.
TNFα circulates as a dimer or trimer of 17kDa subunits. It is synthesized as a extra-cellular membrane associated 26 kDa precursor protein that is further cleaved by a micro-

somal metalloproteinase called TNF-alpha converting enzyme (TACE) to yield the soluble 17 kDa form.[17-19] In its active form TNFα contains 157 amino acids. The genes responsible for TNFα are contained on chromosome 6 in man, within genes of the major histocompatibility complex.[20,21]
The circulating half-life of TNFα is 15-30 min.[22-24] Clearance of TNFα predominantly takes place in the kidney, followed by the liver.[25]
TNFα mediates its activity by binding to specific receptors on the surface of cells. Two distinct TNF-receptors have been identified respectively with a molecular mass of 55 kDa (TNF-R55) and 75 kDa (TNF-R75). Proteolytic cleavage of these membrane-bound TNF-receptors results in soluble TNF-receptors.[26,27] These soluble TNF-receptors compete with the membrane receptors for binding of free TNFα and may therefore have physiologic importance for the regulation of TNF-activity.[28]
Besides the role of inactivation of TNFα the soluble TNF-receptors are also held responsible for the clearance of TNFα.[25]

### *1.2.3 In vitro effects of TNFα*

Early in vitro studies suggested a selective cytotoxic and cytostatic effect for TNFα both in vitro and in vivo. The cytotoxic effect of TNFα was tested on a variety of tumor cell lines.[29-32] From these studies it became clear that the effect of TNFα on tumor cell-lines can be growth-stimulatory, cytostatic (growth inhibitory) or cytotoxic. Further investigations showed that the antiproliferative effect of TNFα is not limited to tumor cells, normal cells can also be inhibited by TNFα. Several mechanisms seemed to be involved in TNF-cytotoxicity.[33]

### *1.2.4 In vivo effects of TNFα*

The murine Meth A sarcoma was the first tumor in which the antitumor effect of TNFα was shown.4,[34,35] The anti-tumor effect, as a hemorrhagic necrosis, was evident within a few hours. Histological evaluation of the hemorrhagic tumor necrosis in murine tumor models suggested a vascular effect of TNFα on the tumor vessels.[36,37] Other studies tried to specify the mechanism of the endothelial and vascular destruction caused by TNFα. The effect of TNFα on proliferation of endothelial cells in mice was shown to be dependent of its local tissue concentration. Low doses (0.01-1.0 ng) of subcutaneous mouse recombinant TNF induced angiogenesis, whereas high doses (1-5 μg) caused inhibition and can even induce destruction of newly formed blood vessels.[38] Tumor associated vasculature showed an enhanced sensitivity for these effects of TNF compared to normal vessels.[39,40]

TNF-induced endothelial cell damage leads to release of von Willebrand factor (VWF).[41] Release of VWF was followed by platelet aggregation and erythrostasis, which results in an impaired blood flow. The impaired blood flow leads to edema, hyperemia, vascular congestion, extravasation of erythrocytes, infiltration of polymorphonuclear neutrophils and hemorrhagic necrosis.[36,42-46]

The importance of the vascular effect of TNFα in the tumor is confirmed by the fact that it has its major effect on larger tumors, with well-developed vasculature, in contrast to small tumors with a poorly developed capillary bed.[47-49]

### *1.2.5 Clinical studies with TNFα*

Systemic administration

Phase I clinical studies in cancer patients were designed to determine the maximum tolerated dose, pharmacokinetics and toxicity of TNFα.

Systemic application of TNFα was limited by unacceptable side-effects, such as hypotension and organ failure. The maximum tolerated dose (MTD) of 400 $\mu g/m^2$ was not able to produce real antitumor effect.[50-56] Results of phase II trials, determining drug effectiveness, were very disappointing. No antitumor effect was observed and treatment with TNFα caused serious toxicity.[57-61]

Intratumoral administration

To avoid systemic toxicity locoregional treatment with TNFα has been tried to achieve tumor exposure to higher TNF concentrations without increasing toxic side-effects. Intratumoral injection of TNFα was not successful: in superficial tumors there was tumor regression, but in deep-seated tumors (pancreas- and hepatocellular cancer and 1 metastatic liver tumor) it resulted in stable disease.[62] In another study 5 out of 14 patients with different advanced solid tumors intratumoral injection of TNFα resulted in a short-lived local tumor regression, implying a rapid development of resistance to recombinant TNFα application.[63] For hepatic metastases 53% stable disease was observed without any responses.[64] Better results were observed in Kaposi sarcoma: 94% tumor regression, 19% complete response.[65] Intratumoral administration of TNFα had the same toxicity profile as subcutaneous and intravenous administration of TNFα, caused by leakage from the tumor to the systemic circulation.[63]

Intracavitary administration

Intraperitoneal administration of TNFα in gynaecological cancer had no tumor regressive effect, but surprisingly it led to disappearance of ascites.[66,67] Abdominal pain was dose-limiting.[68,69] Disappointing was the observation that intraperitoneal administration of TNFα stimulated micro-metastatic implantation in the peritoneum in intraperitoneal human xenograft models.[70]

Intrapleural administration in patients with malignant pleural effusion resulted in 50% of the patients in resolution of pleural fluid.[66] This effect was also observed in a phase I study of our group, in which the effects of intrapleural administration of TNFα in patients with malignant pleural mesothelioma was studied (chapter 5 of this thesis).[71]

Intravesical administration of TNFα in patients with superficial carcinoma of the bladder was well tolerated[72,73] and resulted in a complete response in 4 out of the 15 evaluable patients (26%).[74]

Intra-arterial infusion

Intra-arterial infusion of TNFα has been used in the treatment of liver metastasis and brain tumors. In hepatic arterial infusion an MTD of 150 $\mu g/m^2$ could be achieved. Dose-limiting was severe hypophosphatemia.[75] This MTD was still far from the effective dose. Objective tumor response (partial response) was observed in 2 out of 14 patients with colorectal liver metastasis and 3 patients had a minor response.[76]

Treatment of 20 patients with malignant glioma with intra-arterial injection of TNFα led to 30% objective tumor regression in the group of 10 evaluable patients. In 15 out of the 20 patients some tumor necrosis was observed. The treatment was well tolerated.[77]

Isolated limb perfusion

In 1988 Lejeune and Lienard at the Institut Jules Bordet in Brussels pioneered the idea to use TNFα in the isolated limb perfusion system for the treatment of regionally advanced melanoma and sarcoma.[78] The technique of isolated limb perfusion (ILP) was already an established method in the treatment of in transit metastasized melanoma.[79] With this technique the intravascular concentration of TNFα could be dramatically increased because the ILP system is leakage free and most of the agent is washed out from the limb at the end of the perfusion. ILP with TNFα alone (9 patients) resulted in 2 complete responses (CR), that lasted for 7 months and for other 4 cases a short-lived response: 3 partial responses (PR) and 1 minor response (MR), 3 patients had no response.[78,80]

Since preclinical studies showed a synergistic effect of TNFα with interferon-gamma (IFNγ)[81-83] and with alkylating chemotherapeutic drugs[84-87], a combination triple drug regimen of TNFα plus melphalan and IFNγ was developed. Additionally, ILP was performed under hyperthermia (40°C). An enhancement of the cytolytic activity of TNFα by hyperthermia has been demonstrated both in vitro[88] and in vivo[89,90]. This regimen used for ILP of extremity melanoma resulted in a 100% overall response and a 90% complete response rate (CR).[91-93] In patients with locally advanced sarcoma treated with ILP a major tumor response was seen in 82-92% of patients and made the sarcomas resectable in most cases after perfusion with TNF and melphalan and IFNγ[94] or without IFNγ.[95-97] Final outcome was 28-38% CR, 54-57% PR and 8-22% no change. Limb salvage was achieved in 82-85% of the patients.[94-96,98,99] Independent review committees of the registration file of TNF concluded that in some patients an ILP had not been strictly necessary. Still in 196 patients without any other option it was concluded that limb salvage was achieved in 71% due to a TNF-based ILP.[97] These significant therapeutic improvements have recently resulted in the approval of TNF by the European Medicine Evaluation Agency (EMEA) for advanced sarcomas (Eggermont, 1999).
The isolated limb perfusion system was used as a model to developing new treatment strategies.[100] Because a number of organs can be perfused in a similar fashion isolated lung[101,102], liver[103,104] and kidney[105,106] perfusion models has been developed in animals and also phase I-II trials. Especially for isolated liver perfusion promising results had been attained with response rates of about 70% in patients with colorectal hepatic metastasis and primary malignant liver tumors.[107-110] To reduce the surgical stress (caused by laparotomy to isolate the liver from the systemic circulation) an isolated hypoxic hepatic perfusion using balloon catheters was developed. Using this method makes laparotomy unnecessary.[108,111,112] The most recent developments are the application of TNFα-mutants (that have retained antitumor activity with less toxicity) and encapsulation of TNFα into liposomes which augmented accumulation in tumor tissue.[113-115]

## 1.3 Cytokines and the acute phase response

### *1.3.1 The acute phase response*

The acute phase response (APR) is the answer of the organism to disturbances of its homeostasis due to infection, tissue injury, neoplastic growth or immunological disorders. It consists of a local reaction at the site of injury characterized by a number of responses such as aggregation of platelets and clot formation, dilation and leakage of blood

vessels, and an accumulation and activation of granulocytes and mononuclear cells. These cells are responsible for the release of acute phase cytokines. These mediators act on specific receptors on different target cells leading to a systemic reaction characterized by (among others) fever, leukocytosis, thrombocytosis, anorexia, increases in secretion of glucocorticoids and dramatic changes in the concentration of some plasma proteins. These proteins are named acute phase proteins (APPs).

*1.3.2 History of the acute phase response*

The earliest description of the APR came from the ancient Greeks (cited by Fahraeus).[116] They already observed an increased sedimentation rate of erythrocytes in blood of severely ill patients. As was discovered later, this increase is due to elevated plasma concentrations of fibrinogen and other APPs.[117,118] With the discovery of C-reactive protein (CRP)[119] the search for other acute phase proteins was started. In 1951 Miller et al. showed that the liver is the major organ for the synthesis of acute phase proteins.[120] The fact that the synthesis of APPs takes place in the liver and injury to another part of the body results in an increase of APPs, led to the search for hormone-like mediators. Thirty years later a monocyte-derived polypeptide was described, which was involved in the regulation of APP-synthesis.[121,122] They named this polypeptide hepatocyte-stimulating factor (HSF). HSF was later shown to be identical with interferon-β2, B cell stimulatory factor-2 and hybridoma plasmacytoma growth factor and was named IL-6.[123,124] The production of HSF by human monocytes was found to be regulated by lipo-polysaccharide.[125] Since TNFα and IL-1 were also released by monocytes after exposure to lipopolysaccharide, these cytokines were also investigated for their capacity to stimulate the liver to production of acute phase proteins.[126-129] They were found to stimulate production of a subset of APPs (see below).

*1.3.3 Definition of acute phase proteins*

An acute phase protein has been defined as a protein whose plasma concentration increases (positive APPs) or decreases (negative APPs) by at least 25% during inflammatory disorders.[130] The APR follows a sequential pattern with changes in APPs varying in moment and size of increase. Within the first 24 hours CRP and serum amyloid A (SAA) increase with a magnitude of 1000-fold the initial value. This first wave is followed by a slower one including APPs like α1-acid glycoprotein, α1-antitrypsin and fibrinogen. These APPs increase modestly till about 50% over the initial value (Figure 1).[131]

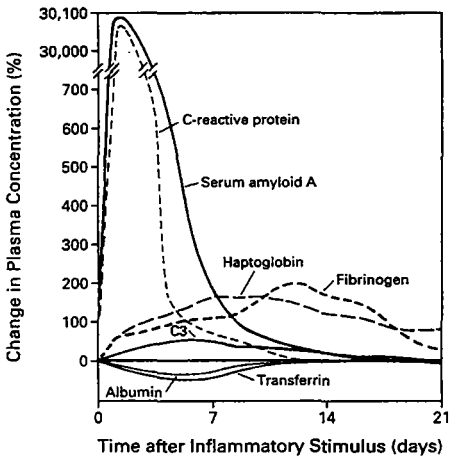


*Fig.1 Sequential acute phase protein patterns following an inflammatory stimulus. From ref.131.*

The acute phase proteins can be divided in 2 subgroups according to the cytokine subsets that regulate APP synthesis in vitro (Table 1).[132-134] Type I APPs are induced by IL-1 type cytokines (TNFα and β and IL-1α and β) and include CRP, SAA, complement factor C3 and α1-acid glycoprotein.[135,136] Type II APPs, including fibrinogen, haptoglobin, α1-antitrypsin, α1-antichymotrypsin and ceruloplasmin were produced in response to the IL-6 family of cytokines (IL-6, leukemia inhibitory factor, IL-11, oncostatin M and ciliary neurotrophic factor.[137-140] The IL-1 type cytokines are synergized by IL-6 in the regulation of type I APPs. So, IL-6 stimulates the full spectrum of APPs in primary human hepatocytes in vitro.[129] The synthesis of the negative APPs (albumin, transferrin, alpha-fetoprotein and α2-HS glycoprotein) is inhibited by both IL-1 and IL-6 type cytokines in vitro.[129,135,141]

This classification is completely based on in vitro studies. Therefore the effect of combinations of cytokines on human hepatoma cell lines has been studied.[141-143] It was concluded that cytokines could have an additive, inhibitory or synergistic effect on each other. From clinical studies in cancer patients it became clear that in vivo hepatocytes are exposed to a mixture of cytokines, rather than to individual mediators.[144-146] Cytokines operate both as a cascade and as a network in stimulating the production of APPs.

*Table 1. Classification and regulation of acute phase proteins, based on in vitro studies*

| APP classification | APP | Regulation |
|---|---|---|
| *Positive APP* | | |
| Type I APP | C-reactive protein | IL-1 type cytokines: |
| | Serum amyloid A | IL-1α and β |
| | α1- acid glycoprotein | TNFα and β |
| | complement C3 | |
| | | *Potentiated by:* |
| | | IL-6 type cytokines |
| | | Glucocorticoids |
| Type II APP | α1-antitrypsin | *IL-6 type cytokines:* |
| | α1-antichymotrypsin | IL-6, IL-11, leukemia |
| | | inhibitory factor, |
| | fibrinogen | ciliary neurotrophic factor, |
| | haptoglobin | oncostatin M |
| | hemopexin | |
| | ceruloplasmin | *Potentiated by:* |
| | | Glucocorticoids |
| *Negative APP* | Albumin | IL-1 and IL-6 type cytokines |
| | Transferrin | |
| | α-fetoprotein | |
| | α2-HS glycoprotein | |

*1.3.4 Regulation of the acute phase response*

The cytokines that are produced during inflammatory processes are the chief stimulators of the production of APPs. These inflammation associated cytokines include IL-6, IL-1β, TNFα, IFNγ and transforming growth factor-β (TGFβ).[147-150] The most important sources of these cytokines are monocytes and macrophages at inflammatory sites. IL-6 is the major regulator of the production of most APPs, whereas the other implicated cytokines influence subgroups of APPs.

Regulation of APP-synthesis occurs mainly by transcriptional control.[132,134,151,152] Post-transcriptional mechanisms may also participate.[153,154] Besides cytokines also other factors influence the APR. Glucocorticoids generally enhance the stimulatory effects of cytokines on the production of APPs.[127,134,155] Additionally glucocorticoids play an important role in the counterregulation of the inflammatory response by inhibition of cytokine-production (see below). Several other hormones like insulin, thyroxin and human growth hormone modulate the effects of cytokines on the production of some APPs.[134,156-158] Thus, the regulation of the APR is very complex, involving cooperative action of several mediators of the synthesis of APPs at different levels.

The majority of studies have concentrated on examination of the mechanisms of initiation and propagation of the APR. Regarding the termination of the APR little is known. One might not necessarily expect an inhibitory mechanism to be required because of the short half-life of many of the cytokines involved in the APR. The process would be turned off by extinction of the initiation-mediators.

However much more mediators could be involved in the resolution of the APR (table 2). For instance, excess of pro-inflammatory cytokines are removed by soluble cytokine receptors (like soluble TNF-receptors) and receptor antagonists (such as IL-1 receptor antagonist).[159,160] Furthermore, synthesis of proinflammatory mediators and cytokines can be blocked by glucocorticoids[161,162], some non-steroidal anti-inflammatory drugs (suppressing cyclooxygenase) and by specific inhibitors of cytokine induction[162]. In addition inhibitory functions for some cytokines, including IL-4 and IL-10 have been described.[134] Finally, an anti-inflammatory and immunosuppressive effect was suggested for IL-6 and some of the IL-6 regulated APPs.[163,164] IL-6 was found to moderate levels of TNFα and IL-1, to induce glucocorticoid release and soluble TNF-receptors and IL-1 receptor antagonists. IL-6 regulated APPs were shown to induce IL-1 receptor antagonist synthesis.

*Table 2. Resolution of the APR*

| Resolution of the APR | |
|---|---|
| • Short half-life of initiating cytokines | [162] |
| • Production of anti-inflammatory cytokines (IL-4, IL-10) | [134] |
| • Removal of pro-inflammatory cytokines by soluble cytokine-receptors and receptor-antagonists | [159,160] |
| • Synthesis of pro-inflammatory mediators blocked by glucocorticoids, NSAIDs, specific inhibitors cytokine-induction | [161,162] |
| • Anti-inflammatory and immunosuppressive effect of IL-6 and some IL-6 regulated APPs | [163,164] |

Normally, the APR subsides within 24-48 hours and after a few days the organism returns to normal function. However, the APR can be prolonged and converted in a chronic phase of inflammation. The mechanism of conversion from APR to chronic inflammation is still poorly understood.[165,166] One of the aims of this thesis is to get more insight in the role of the APR in chronic inflammation (chapter 4 & 6).

### *1.3.5 Interferon-alpha and the acute phase response*

The interferons (IFNs) had been discovered in 1957 by Isaacs and Lindenmann.[167] They observed that chick chorioallantoic membranes incubated with a heat-inactivated influenza virus produced a substance called IFN. This substance was found to cause resistance to infection by live virus.[168] Further investigations pointed out that there were different IFN-proteins: type I consists of IFNα and IFNβ and type II represents IFNγ. IFNs bind to specific cell surface receptors as the first step in the expression of their biological activity. Because IFNα and IFNβ share components of the same receptor, they are named type I IFNs. IFNγ uses a separate receptor system and is referred to as a type II IFN.[169,170]

Under normal conditions IFNα is not detectable in the systemic circulation.[171,172] Contrary to many other cytokines type I IFNs can be produced by nearly all sorts of cells.[173,174] Production of IFNα is mostly induced by viral infection.[175-177] Viral structures were shown to interact with intracellular signalling pathways to induce expression of the type I IFNs.[178] IFNs play a dominant role in nonspecific inhibition of virus replication.[173,179,180]

Further investigations showed that besides its antiviral effect IFNα had also an antitumor effect in experimental tumors.[181,182] After its cloning in 1981 large quantities of

recombinant IFNα became available, facilitating the evaluation of the various properties of IFNα.[183-185] IFNα was the first cytokine that was cloned. In addition to its effects in viral diseases and malignancies IFNα was also found to be effective in the treatment of inflammatory, fibrotic and angiogenic diseases.[186]

IFNα and cytokine-response

It was suggested that some of the therapeutic and toxic effects of IFNα might be caused by the induction or inhibition of other cytokines. In vitro experiments showed that IFNα mainly has a stimulatory effect on the production of other cytokines like IL-1, IL-6 and TNFα.[187]

Only one study described the effect on other cytokines of a single dose of IFNα in healthy men.[188] All other studies are more complex because of the effect of the disease treated by IFNα. Corssmit et al. found pro-inflammatory effects as well as anti-inflammatory effects of IFN-alpha within the cytokine network: IFNα induced release of the pro-inflammatory cytokines IL-6 and IL-8 and of the anti-inflammatory cytokine IL-10 and of the soluble TNF receptors p55 and p75.[188,189] Concentrations of TNFα and IL-1 remained below the detection limit.

In patients with chronic hepatitis C IFNα induced production of IL-6 by peripheral blood mononuclear cells (PBMC), regulated at messenger RNA level. TNFα and IL-1β were not elevated.[189-191] Chronic administration of IFNα during 6 months resulted in a decrease of IL-6 levels in complete responders and an increase in partial and non-responders.[192] Pre- and post-treatment levels of IL-6 were higher in hepatitis patients compared to healthy controls, indicating that both viral-induced inflammation and treatment with IFNα influenced IL-6 concentrations. In Behcet's disease IFNα brought about a decrease in levels of TNFα, which were elevated before treatment, without changing IL-2 levels (not elevated before treatment).[193] The patients experienced also clinical improvement, indicating that the decrease in TNFα most possibly was caused by the diminution in disease-activity. So, IFNα seems to induce both pro- and anti-inflammatory cytokines indicating a complex role within the cytokine network.

IFNα and acute phase proteins

In vitro studies describing the effect of IFNα on acute phase proteins could not be found. In healthy persons IFNα induced a decrease in the serum iron concentration and transferrin saturation with a concomitant increase in ferritin.[194] CRP remained below detection limit.[189,194]

In some patient groups treated with IFNα besides clinical evaluation also parameters of the APR were measured. Treatment with IFNα in patients with rheumatoid arthritis resulted in a significant improvement in the patients' joint score, in CRP-levels and platelet count.[195] In patients with chronic active Crohn's disease administration of IFNα did not show any beneficial effect on IL-6 or APP-levels and on endoscopic activity.[196] Finally, a case-report was published describing a patient with chronic hepatitis C and liver hemosiderosis who was treated with long term IFNα-therapy. At the beginning of the treatment an increase of ferritin was observed, followed by a decrease most possibly due to improvement of the clinical situation.[197] From these studies it can be concluded that IFNα seemed to have a stimulatory effect on ferritin-levels, without affecting other APPs. In chapter 6 this conclusion was tested in melanoma-patients treated with IFNα.

### 1.4 Aims and outline of the thesis

Patients who underwent ILP with TNFα and melphalan enabled us in a unique way to investigate effects of administration of TNFα on the immune response in humans. In this thesis the in vivo effects on the inflammatory response and immunologic homeostasis mechanisms are discribed. Effects on various components of the immune system: effects on immune cells, secondary cytokine responses- and kinetics of the acute phase reaction in response to TNFα are described and discussed. Long-term effects of TNFα and IFNα on the immune response were determined in mesothelioma-patients treated with repeated intrapleural administration of TNFα and in patients with melanoma who were treated with IFNα. The goal of these endeavours is to obtain a better understanding of how to use TNF best with regards to the TNF-associated toxicity, that so far has greatly limited its role in the clinical setting, how to overcome these limitations and create the possibility to utilize TNF in additional ways in the treatment of cancer patients.

In chapter 2 the number of monocytes and lymphocytes was investigated in response to systemic leakage of TNFα during and after ILP. We also determined the function of the remaining monocytes and lymphocytes by measuring production of cytokines after stimulation in whole blood cell culture. The results in ILP-patients were compared to the results in patients undergoing various surgical operations to study the effects of surgery and anaesthesia.

In addition to these results we specified in chapter 3 the effect of TNFα on number and function of T lymphocytes and the T helper 1/ T helper 2 balance. Two methods of determination of T cell function were compared: intracytoplasmic staining of cytokines

(IL-2, IL-4 and IFNγ) after stimulation of cultured peripheral blood mononuclear cells and production of the same cytokines in the whole blood cell culture system.
In chapter 4 the clinical course after exposure to very high systemic levels of TNFα in patients undergoing ILP complicated by high leakage from the perfusate was described. The patterns of cytokines and the acute phase response in response to high levels of TNFα were compared to measurements in patients who underwent ILP without detectable leakage.
The effects of prolonged presence of TNFα on other cytokines and the acute phase response were investigated in mesothelioma-patients treated with intrapleural administration of TNFα (chapter 5). Furthermore the effects of repetitive administration of TNFα were determined.
With the obtained knowledge about the APR we ended with a study of the acute phase response in melanoma-patients treated with prolonged administration of IFNα (chapter 6). In this study our hypothesis that the hyperferritinaemia in patients with adult onset of Still's disease could be a direct effect of IFNα was tested.

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# CHAPTER 2

## EFFECTS OF ISOLATED LIMB PERFUSION WITH TUMOR NECROSIS FACTOR-ALPHA (TNFα) ON THE FUNCTION OF MONOCYTES AND T-LYMPHOCYTES IN PATIENTS WITH CANCER

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## ABSTRACT

The objective of the present study was to investigate the effects of isolated limb perfusion (ILP) with tumor necrosis factor-alpha (TNFα) and melphalan in patients with cancer on, first, plasma levels of cytokines, second, systemic monocyte and T-lymphocyte distribution and third, the ability of mononuclear cells to produce cytokines upon stimulation in vitro.

Six patients undergoing an ILP were entered into the study (group 1). In addition, patients undergoing a major surgical operation (group 2), minor operation (group 3) as well as healthy volunteers (group 4) were included as a control groups. Sensitive enzyme-linked immunosorbent assays (ELISAs) were used to measure TNFα and interleukin-6 (IL-6) plasma levels at various time points during and after operation. Furthermore, the percentage of monocytes and T-lymphocytes was determined in all studied groups using FACScan. In addition, cytokine production upon stimulation with lipopolysaccharide (LPS) and combination of anti-CD3/anti-CD28 monoclonal antibodies in whole-blood cultures was investigated.

Increased plasma levels of TNFα and IL-6 in patients undergoing ILP were observed, but only IL-6 appeared to be increased in patients treated with a major operation. No significant fluctuations were found in the other groups studied. Concerning the number of monocytes, a significant decrease was observed only in patients treated with ILP. Furthermore, a decreased production of TNFα, IL-6 and IL-8 upon various types of stimulation in vitro was found in those patients, but also after a major operation.

In conclusion, the results of present study show increased plasma levels of cytokines in patients treated with ILP and major operation. Furthermore, a decrease in numbers of monocytes in the circulation and the ability of mononuclear cells to produce cytokines in vitro may be induced by administration of TNFα in ILP. Although similar results were found in patients treated with major operation, the underlying mechanisms of this phenomenon remains to be elucidated.

## INTRODUCTION

Isolated limb perfusion (ILP)[1] with cytostatic drugs has been demonstrated to be a successful treatment of regionally advanced melanoma. One of the advantages of this treatment modality is that up to 50 times higher concentrations of cytostatic drug can be delivered with minimal systemic side-effects.[2] The current treatment regimen for regionally advanced melanoma[3] and sarcoma[4] consists of high dose tumor necrosis factor-alpha (TNFα) combined with the melphalan in condition of mild hyperthermia. During or after ILP, TNFα may leak from the isolated limb circuit into the circulation. The effects of TNFα leakage in patients has been investigated previously .[5,6]

The aim of the present study was to investigate the effects of systemic leakage of TNFα during and after ILP in patients with cancer. First, the level of TNFα and the secondary induction of cytokines (interleukin-6) was measured in consecutive plasma samples during and after ILP. Second, since TNFα has been reported to stimulate the adhesion of mononuclear cells to the vessels endothelium[1,4,7,8], possible alterations in the number of circulating monocytes and lymphocytes were investigated during the ILP with TNFα. Furthermore, the function of peripheral blood monocytes and lymphocytes was studied by measuring the cytokine production in vitro. Different stimuli, such as lipopolysaccharide (LPS) and combination of anti-CD3/anti-CD28 monoclonal antibodies were used to stimulate cytokine production by monocytes/macrophages and T-lymphocytes, respectively.[9,10] To exclude effects of surgery and anaesthesia we also examined patients undergoing various surgical operations.

## MATERIALS AND METHODS

*Patients and controls*

Six patients undergoing an isolated limb perfusion (ILP) with TNFα and melphalan as treatment of malignant melanoma or sarcoma entered into this study (group 1). To investigate the effect of operation-duration and effect of anaesthesia these patients were compared with six patients undergoing a different operation: 3 patients were treated with a major abdominal operation (group 2) and 3 patients with a minor limb and/or chest operation (group 3). To exclude the effect of diurnal variation on the results, blood samples were sequentially obtained over the day from 6 healthy volunteers (group 4). The demographical and clinical characteristics of the patients are summarized in Table 1.

*Table 1. Demographical and clinical data*

| *Patientno.* | Age (years) | Sex | Group | Diagnosis | Treatment | Operation duration (h) |
|---|---|---|---|---|---|---|
| 1 | 52 | M | 1 | Melanoma | ILP | 3.5 |
| 2 | 63 | M | 1 | Melanoma | ILP | 3.5 |
| 3 | 51 | F | 1 | Sarcoma | ILP | 3.5 |
| 4 | 82 | F | 1 | Sarcoma | ILP | 3.5 |
| 5 | 82 | F | 1 | Sarcoma | ILP | 3.5 |
| 6 | 64 | F | 1 | Melanoma | ILP | 3.5 |
| 7 | 66 | M | 2 | Strangulation ileus | Laparotomy | 1 |
| 8 | 26 | M | 2 | Hepatocellular ca. | Laparotomy | 3 |
| 9 | 66 | F | 2 | Ovarial ca. | Secondary debulking | 1.5 |
| 10 | 41 | F | 3 | Sarcoma (limb) | Resection | 1 |
| 11 | 54 | M | 3 | Pneumonia | Open lung biopsy | 1.5 |
| 12 | 59 | M | 3 | Squamous cell ca. | Limb amputation | 1 |

*ILP, isolated limb perfusion; ca., carcinoma. Group 1 consists of ILP-patients. Group 2 consists of patients undergoing a major operation. Group 3 consists of patients undergoing a minor operation.*

*Treatment design of isolated limb perfusion*

The details of the surgical procedures have been published previously.[4] Briefly, ILP consisted of a 90 minutes long perfusion with 3-4 mg of recombinant human TNFα (Boehringer Ingelheim, Germany) and 10-13 mg/l perfusion tissue of melphalan (Alkeran) (Burroughs Welcome, London, UK) at mild hyperthermia (40°C). The perfusate was composed of a priming volume of 700-850 ml consisted of 400-500 ml blood (50% red blood cells, 50% plasma), 200-400 ml of 5% dextran 40 in glucose, 5% (Isodex, Pharmacia, Uppsala, Sweden) Haemaccel, 10-30 ml of 8.4% sodium bicarbonate and 0.5 ml of 2500-5000 IU heparin. TNF was injected as a bolus into the arterial line provided limb tissue temperature was > 38°C. Melphalan was administered 30 minutes later at limb temperatures between 39 and 40°C. At the end of perfusion, the limb was washed twice with at least 1 l of Haemaccel and 1 l of 6% dextran 70 (Macrodex, Pharmacia, Upsala, Sweden).

*Blood samples*

Venous blood samples were collected at five time points: pre-operative (patient already under anaesthesia), just before ILP and 95 minutes (5 minutes after completion of the washout procedure at the end of the perfusion and the release of the tourniquet) and 3 and 24 hours after the ILP. The control patients (group 2 and 3) and healthy controls (group 4) were sampled at the same time points. The samples were kept at room temperature and used within 7 hours. Blood (10 ml) was collected in evacuated blood collection tubes (Venoject, Terumo, Belgium) containing sodium heparin (150 USP units) and was used for whole blood cell culture experiments. The remaining blood was centrifuged and plasma was stored at -70°C for later cytokine analysis. For measuring the percentage of monocytes and lymphocytes in the circulation, peripheral blood was collected in EDTA-tubes (Vacutainer, Becton Dickinson, France).

*Monocyte and T-lymphocytes distribution*

EDTA-blood (100 µl) was stained with conjugated monoclonal antibodies: phycoerythrin (PE)-labeled anti-CD14 and fluorescein isothiocyanate (FITC) -labeled anti-CD3 (Becton Dickinson) to detect monocytes and T-lymphocytes, respectively. At the same time erythrocytes were removed by lysis through addition of FACS Lysing Solution to the tubes. After incubation for 30 minutes at 20°C and washing twice with phosphate buffer (PBS with 0.1% azide) the samples were analyzed with a FACScan flow cytometer (Becton Dickinson). The percentage of CD14+ (monocytes) and CD3+ (lymphocytes) cells of 5000 counted cells was calculated.

*Whole-blood cultures*

Whole-blood cell cultures were performed in flat-bottom micro-titer plates (Nunc, Kamstrup Denmark). Heparinized venous blood was diluted 1:10 with Iscove's modified Dulbecco's medium (IMDM) supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). The monocytes of diluted blood (100 μl) were stimulated with 4 or 62 pg/ml LPS (lipopolysaccharide, Escherichia coli, Sigma, St.Louis, MO, USA). To stimulate T-lymphocytes combination of anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml) was added to the cultures. Incubation of the whole-blood cell cultures was performed at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After 16 and 72 hours of incubation with LPS or anti-CD3/anti-CD28 respectively, the supernatant was harvested and stored at -70°C until cytokine analysis was performed.

*Cytokine assays*

IL-6. Levels of IL-6 were measured using enzyme linked immunosorbent assay (ELISA) as described previously [11] and expressed in pg/ml. Briefly, flat bottomed microtiter plates were coated over night with purified monoclonal antibody (mAb) against IL-6 (CLB-IL6/16). After washing, serial dilutions of IL-6-containing samples were added. Bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. The lower detection level of this assay was 1 pg/ml and normal healthy controls were below 10 pg/ml.

IL-8. IL-8 was measured by ELISA as described previously.[12] Briefly, a coat of CLB-IL8/1 mAb was applied overnight at a concentration of 1 μg/ml and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8, also at 1 μg/ml. The lower detection limit of this ELISA was about 5 pg/ml of serum. Values of IL-8 for healthy controls determined with this ELISA were below 20 pg/ml.

TNFα. The TNFα-specific ELISA was described earlier.[13] Flat-bottomed 96-wells plates were coated with mAb (18b) anti-hu-TNFα (Hoffman-La Roche, Basel, Switzerland). Biotinylated sheep anti-TNFα was used as a second step. The detection limit of the assay was 10 pg/ml.

*Statistics*

The two-sample Wilcoxon test for unpaired data was used to analyze the differences between groups.

## RESULTS

*Cytokine plasma levels*

At the time point 95 minutes after the perfusion, patients treated with ILP (group 1) showed a significant ($p<0.001$) increase in plasma TNFα, which returned to pre-operation value 3 hours post-operatively (Figure 1). No measurable TNFα was detected in the other studied groups. Concerning IL-6, the highest levels were measured 95 minutes and 3 hours after operation for group 2 and 1, respectively (Figure 1). Patients of group 3 and 4 had no measurable plasma IL-6 (data not shown).

*Monocytes and T-lymphocytes distribution*

At time points 95 minutes and 3 hours after ILP (group 1) a significant lower percentage of monocytes ($p<0.05$) were found in comparison with the pre-operative value (Figure 2A). At day 1 the percentages of monocytes in blood samples of patients from group 1 returned to the pre-operative value. Furthermore, there was a tendency towards decreased percentage of T-lymphocytes in the circulation but the difference as compared with pre-operative values did not reach significance. In the blood samples of group 2, 3 and 4 no significant fluctuations in percentages of monocytes and lymphocytes were found (Figure 2B).

*Whole blood cultures*

*Cytokine-production by monocytes*

Decreased monocytes TNFα production after stimulation with LPS in vitro was observed in patients undergoing an ILP (group 1) or major operation (group 2) as compared with patients undergoing a minor operation (group 3) and healthy controls (group 4) (Table 2). Three hours after operation in both group 1 and group 2, the TNFα production in vitro was completely depressed in comparison with pre-operative production ($p<0.01$). The next morning the ability of monocytes to produce TNFα upon stimulation with LPS was restored. Similar results were obtained for IL-6 production (Table 2).

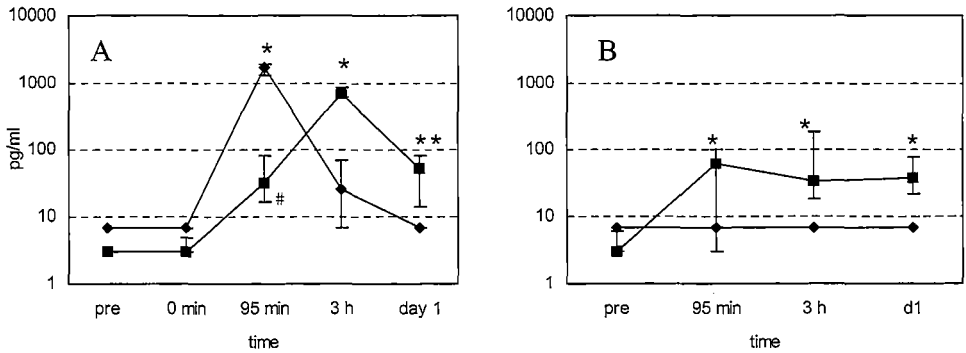


*Figure 1. Median (with range) TNFα (◆) and IL-6 (■) plasma levels in (A) patients treated with isolated limb perfusion (ILP) and (B) patients undergoing a major operation.*
**p<0.001, ** p<0.01, # p<0.05 compared with pre-operative time point.*
*Pre, pre-operative; 0 min; just before perfusion; 95 min, 95 minutes after the start of perfusion/ operation; 3 h, 3 hours after operation; day 1, next morning.*

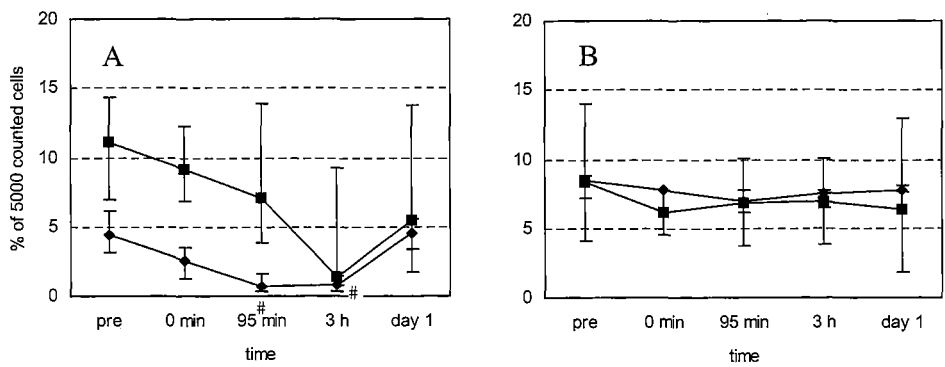


*Figure 2. Median (with range) percentage of peripheral blood monocytes (◆) and lymphocytes (■) in patients treated with isolated limb perfusion (A) and healthy volunteers (B). # p<0.05 compared with pre-operative time point. Pre, pre-operative; 0 min; just before perfusion; 95 min, 95 minutes after the start of perfusion/operation; 3 h, 3 hours after operation; day 1, next morning.*

*Table 2. LPS-stimulated cytokine production (TNFα and IL-6) in whole blood cell cultures in patients undergoing an ILP, a minor/major operation and healthy volunteers*

| TNFα (pg/ml) production upon stimulation with LPS | | | | |
|---|---|---|---|---|
| Timepoints | ILP (n=6) | Major operation (n=3) | Minor operation (n=3) | Controls (n=7) |
| LPS 4 pg/ml | | | | |
| Before | 426 (66-1143) | 547 (359-865) | 948 (688-2004) | 757 (143-1064) |
| 0 min | 76 (29-479) * | ND | ND | 623 (469-865) |
| 95 min | 68 (21-682) * | 371 (84-405) *† | 896 (512-1481) | 673 (573-959) |
| 3 h | 11 (7-241) *† | 100 (62-133) *† | 585 (416-1075) | 817 (420-1573) |
| Day 1 | 77 (58-666) * | 241 (240-398) | 401 (290-857) | 433 (161-1650) |
| LPS 62 pg/ml | | | | |
| Before | 569 (233-699) | 736 (477-906) | 835 (541-1406) | 889 (369-1246) |
| 0 min | 135 (50-206) * | ND | ND | 946 (774-1293) |
| 95 min | 66 (7-730) * | 542 (90-547) *† | 982 (569-1116) | 1112 (605-1263) |
| 3 h | 22 (7-352) *† | 135 (69-147) *† | 502 (443-1525) | 1054 (666-1515) |
| Day 1 | 292 (107-715) * | 380 (326-586) | 675 (293-926) | 950 (313-1398) |
| | IL-6 (pg/ml) production upon stimulation with LPS | | | |
| Timepoints | ILP (n=6) | Major operation (n=3) | Minor operation (n=3) | Controls (n=7) |
| LPS 4 pg/ml | | | | |
| Before | 1687 (247-2903 | 1345 (1176-1370) † | 2384 (1399-2662) | 1257 (333-2431) |
| 0 min | 677 (7-1461) | ND | ND | 1243 (741-1485) |
| 95 min | 43 (7-1280) *† | 832 (295-1256) † | 1886 (1623-2201) | 1359 (1002-2850) |
| 3 h | 123 (53-355) *†‡ | 537 (324-837) *† | 1565 (1434-3142) | 1483 (657-3037) |
| Day 1 | 765 (235-2385) | 1243 (1234-1340) | 1766 (830-2924) | 1307 (408-2243) |
| LPS 62 pg/ml | | | | |
| Before | 1940 (673-3311) | 1801 (1521-1992) | 2469 (1476-2506) | 1576 (840-1974) |
| 0 min | 1129 (269-2051 | ND | ND | 1511 (1145-1683) |
| 95 min | 72 (45-1165) *† | 1030 (230-1580) | 1980 (1509-1998) | 2048 (1151-2924) |
| 3 h | 219 (73-313) *†‡ | 641 (285-1278) *† | 1653 (1617-3598) | 1506 (1427-2518) |
| Day 1 | 2020 (738-2811) | 1776 (1143-2281) | 2181 (2095-3063) * | 1637 (979-2220) |

*Results are expressed as median (range). LPS, lipopolysaccharide; ILP, isolated limb perfusion; timepoints: before, pre-operative; 0 min, just before perfusion; 95 min, 95 minutes after the start of perfusion; 3 h, 3 hours after the end of operation; day 1, next morning. * Patients compared with healthy control subjects (p<0.05).*

*† Patients compared with patients undergoing a minor operation (p<0.05). ‡ ILP-patients compared with patients undergoing a major operation (p<0.05).*

*Cytokine production by T-lymphocytes*

T-lymphocyte function of different groups of patients, as measured by IL-8 production upon stimulation with anti-CD3/anti-CD28 in vitro, is shown in Table 3. IL-8 production in groups 1 and 2 was significantly lower (p<0.05) than in groups 3 and 4. Particularly 3 hours after operation, production of IL-8 upon stimulation was hardly provoked. Furthermore, IL-8 production in group 3 was significantly lower (p<0.05) in comparison with healthy control subjects (group 4) as measured at time point 3 hours after operation. The healthy controls showed a constant production of IL-8 at different time points.

*Table 3. Cytokine production (IL-8) in whole blood cell culture after stimulation with anti-CD3/anti-CD28 in ILP patients, patients undergoing a minor/major operation and healthy controls*

| | IL-8 (ng/ml) production upon stimulation with anti-CD3/anti-CD28 | | | |
|---|---|---|---|---|
| Timepoints | ILP (n=6) | Major operation (n=3) | Minor operation (n=3) | Controls (n=7) |
| Before | 58 (21-270) | 32 (28-33) | 28 (24-96) | 41 (19-286) |
| 0 min | 26 (0.5-144) | ND | ND | 93 (29-884) |
| 95 min | 2.0 (0.2-30) * | 21 (11-44) | 24 (11-41) | 37 (23-296) |
| 3 h | 0.5 (0.2-7.7) *† | 2.9 (0.6-11) *† | 16 (15-21) * | 44 (22-190) |
| Day 1 | 4.7 (3.4-33) * | 8.0 (2.3-67) | 28 (13-50) | 40 (20-124) |

*Results are expressed as median (range). ILP, isolated limb perfusion; timepoints: before, pre-operative; 0 min, just before perfusion; 95 min, 95 minutes after the start of perfusion; 3 h, 3 hours after the end of operation; day 1, next morning. * Patients compared with healthy control subjects (p<0.05). † Patients compared with patients undergoing a minor operation (p<0.05).*

## DISCUSSION

In the present study the effects of ILP with TNFα were studied on the plasma cytokine profile (IL-6, TNFα), the number of monocytes and lymphocytes measured in the circulation, as well as cytokine production (TNFα, IL-6, IL-8) ex vivo, measured by the whole blood cell cultures. These results were compared with patients undergoing various operations. Eventual diurnal effects on the results were studied by following healthy control subjects.

Increased plasma-levels of TNFα and IL-6 observed in the ILP-patients were described previously.[5,14,15] In the present study IL-6 was also increased in patients undergoing ILP and in patients treated with major operation. That is in accordance with the observations of Glaser et al.[16] in patients undergoing a conventional cholecystectomy, but much lower than described by Cruickshank et al.[17] in a study of serum IL-6 levels after surgery of various degrees of severity. The rise in IL-6 plasma levels in ILP-patients may be caused by the preceding TNF-peak. In the patients undergoing a major operation, we could not measure TNFα in plasma, whereas IL-6 was certainly detectable after operation (Figure 1). A possible explanation may be that local release of endotoxin after bowel handling during intra-abdominal surgery stimulates systemic release of IL-6.[17]

It has been suggested previously that systemic administration of TNFα may influence monocyte and T lymphocyte distribution in the circulation.[18,19] In the present study the greatest changes in cell-distribution were observed in patients undergoing an ILP. The percentage of monocytes decreased, with the maximum at 3 hours after operation, but rebounded to the pre-operative value within 24 hours. However, the T-lymphocytes only showed a tendency to decrease in comparison with the pre-operative values. These observations may be caused by increased margination of lymphocytes and monocytes as a result of their adherence to the vessel walls and/or migration into the tissues under the influence of the circulating cytokines.[7,8,19,20] Recent observations in our patients showed hardly any alteration in the cell distribution in the perfusate during operation. So, although sequestration took place during operation, it cannot be excluded specifically in the perfused limb or on the total body. Since no fluctuations in cell distribution were observed in patients undergoing major or minor operation, the effect of surgery or anaesthesia on monocyte decrease in patients undergoing ILP seems less likely.

Decrease in production of cytokines after major operation as measured by a whole blood cell cultures was described earlier by Pirenne et al.[21] This is in accordance with the present findings in which decreased production of IL-6, TNFα and IL-8 upon stimulation

in vitro during and after ILP and major operation was found. However, no such effect was observed in patients undergoing a minor operation or in normal control subjects. The underlying mechanism is not completely understood. In vitro studies showed that the synthesis of cytokines can be blocked by corticosteroids.[22] A possible role in the observed effects of ILP and major operation, may be played by cortisol, since the tissue-injury (in patients with major operation) and leakage of TNFα (in patients with ILP) may result in release of cortisol.[6,21] Furthermore, the decrease in the number of monocytes, as observed in the present study, may also play a part in the drop in cytokine-production. However, by expressing the cytokine production per 1000 monocytes, a depressed IL-6 and TNFα production was still calculated (data not shown). The shift of lymphocyte subpopulations during operation was reported previously.[23] Relation between cortisol and lymphocyte number was described by Hamid et al.[24], where an increase in cortisol was followed by a decrease in the number of lymphocytes in the circulation. Decreased IL-8 production by lymphocytes in vitro might represent a reduced function of those cells, since no significant decrease in the lymphocyte number during ILP and various surgical operations was found in the present study.

In conclusion, this study describes the cytokine profiles after different forms of surgical interventions. Patients undergoing an ILP with TNFα and melphalan showed elevated levels of TNFα and IL-6 in the circulation. Only in this group of patients was TNFα measurable in the circulation. However, in patients undergoing a major opera-tion IL-6 was also detected, perhaps caused by the release endotoxins during the intra-abdominal operation. The effects of anaesthesia and/or duration of the operation on present results may, to some extend, be excluded as no effects on monocyte and T-lymphocyte distribution and function were found in patients treated with minor operation. During, but also shortly after operation, monocytes and T-lymphocytes showed a decreased ability to produce cytokines. Thus, TNFα appears to have a profound effect on T cell and monocyte function. This modulation remains to be elucidated.

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# CHAPTER 3

## EFFECTS OF TUMOR NECROSIS FACTOR-ALPHA (TNFα) AND MELPHALAN ON THE CYTOKINE PRODUCTION OF CIRCULATING T-CELLS IN PATIENTS WITH CANCER

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## ABSTRACT

**Background:** The objective of the present study was to investigate the effects of isolated limb perfusion (ILP) with tumor necrosis factor-alpha (TNFα) and melphalan on circulating T-cells from cancer patients, using two different methods.

**Patients and methods:** Eight patients undergoing an ILP entered the study. At first, the number of T-cells at several time-points was determined using FACScan. Subsequently, production of interferon-gamma (IFNγ) (T-helper 1) and interleukin-4 (IL-4) (T-helper 2) was measured at the intracytoplasmic level after stimulation with phorbol 12-myrastate 13-acetate (PMA) and ionomycin. IFNγ, IL-4 and IL-2 (also T-helper 1) production in the whole-blood cell culture system was then determined after stimulation with a combination of anti-CD3/anti-CD28 monoclonal antibodies.

**Results:** An enormous decrease in the number of circulating T-cells was observed. In the remaining T-cell population cytokine-production (IL-2, IL-4, IFNγ) was depressed, showing the same pattern in both methods. No difference could be detected between the effect of TNFα and melphalan on Th1- and Th2-cells.

**Conclusions:** The results demonstrate that TNFα and melphalan reduced the number of circulating T-cells and at the single-cell level decreased the cytokine-production in the remaining circulating T-cells. No selective effect of TNFα on Th1- or Th2-cells could be detected. If the impaired T-cell function is representative for all T-cells remaining in the systemic circulation, this could help to explain the tolerability of high TNF concentrations after ILP, perhaps by decreasing the synthesis and production of T-cell derived cytokines.

## INTRODUCTION

At present, TNFα is used in the treatment of patients with cancer. The current treatment regimen in patients with regionally advanced melanoma and sarcoma consists of isolated limb perfusion (ILP) with TNFα and melphalan under mild hyperthermia.[1-3] This treatment enables us to study the effect of TNFα on the immune system in these patients.
Until now, only a few studies of the effect of TNFα in normal subjects have been carried out. It has been shown in healthy humans that TNFα induces the release of mainly pro-inflammatory cytokines accompanied by activation and degranulation of leukocytes. Lymphocyte-number was decreased by TNFα-infusion.[4] Furthermore, TNFα activates coagulation via the extrinsic pathway, leading to the formation of thrombin[5], and fibrinolysis (measured by plasminogen activator (PA), u-PA, t-PA and PAI-1)[6].
In cancer-patients treated with TNFα intravenously, stimulation of T-cells and monocytes has been observed, as reflected by an increase in soluble interleukin-2 (IL-2) receptors and circulating neopterin respectively.[7] In another study, induction of IL-6, C-reactive protein (CRP) and cortisol was observed after intravenous administration of TNFα in cancer patients.[8]
We reported previously on the induction of the acute phase protein reaction and stimulation of the immunoglobulin synthesis in cancer patients treated with an ILP with TNFα and melphalan.[9] Fibrinolysis was initially activated, induced by leakage of TNFα from the perfusion system, and was subsequently inhibited by PAI-1.[10]
The aim of our study was to investigate the effect of TNFα on circulating T-cells. Our main interest in this study was whether in this acute situation, i.e. in patients treated with TNFα intravenously, alterations in the T-helper 1 (Th1)/ T-helper 2 (Th2) balance were induced. This question is related to observations in patients with rheumatoid arthritis, in whom increased levels of TNFα and a disturbed Th1/Th2 balance have been observed.[11]
To obtain insight in the Th1/Th2 balance in our ILP-patients we used two methods: intracytoplasmic IFNγ and IL-4 staining after culturing of peripheral blood mononuclear cells (PBMCs) derived during and after ILP and production of these cytokines in the whole-blood cell culture system.

## PATIENTS AND METHODS

*Patients and treatment*

Blood samples were obtained from patients undergoing an ILP with TNFα and melphalan as a treatment for melanoma and sarcoma. Clinical and demographic characteristics are summarized in table 1.

The procedure of ILP has been described previously.[3] Briefly, ILP consisted of a 90 minutes long perfusion with 3-4 mg of recombinant human TNFα (Boehringer Ingelheim, Germany) and 10-13 mg/l perfusion tissue of melphalan (Alkeran) (Burroughs Wellcome, London, UK) at mild hyperthermia (39 to 40°C). The perfusate was made up of a priming volume of 700-850 ml, which consisted of 400-500 ml of blood (50% red blood cells, 50% plasma), 200-400 ml of 5% dextran 40 in glucose 5% (Isodex, Pharmacia, Uppsala, Sweden), 10-30 ml of 8.4% sodium bicarbonate and 0.5 ml of 2500-5000 IU heparin. TNFα was injected as a bolus into the arterial line provided that the limb tissue temperature was > 38°C. Melphalan was administered 30 minutes later at limb temperatures between 39 and 40°C. At the end of perfusion, the limb was washed with at least 2 liters of 6% dextran 70 (Macrodex, Pharmacia, Uppsala, Sweden). During perfusion, leakage from the perfusate to the systemic circulation was monitored by the use of radioactive human serum albumin (injected in the perfusate) and a gamma-detector above the heart.

*Blood samples*

Venous blood samples were collected at five time points: pre-operative, just before ILP, and at 95 minutes (5 minutes after completion of the washout procedure at the end of perfusion and the release of the tourniquet), and 3 and 24 hours after ILP. In addition, three samples were taken from the perfusate: just before TNFα was added to the perfusate, halfway through perfusion and at the end of the perfusion (just before the washout).

Blood for the whole-blood cell culture and the intracytoplasmic cytokine staining was collected in 10 ml evacuated blood collection tubes (Venoject, Terumo, Belgium) containing sodium heparin (150 USP units). For determining changes in the cell population, blood was collected in EDTA-tubes (Vacutainer, Becton Dickinson, France).

*Cell-number and -distribution*

EDTA-blood (100 μl) was stained with fluorescein isothiocyanate (FITC)-labelled anti-

CD3 (Becton Dickinson) to detect T-lymphocytes. At the same time, erythrocytes were removed by lysis by the addition of FACS Lysing Solution (Becton Dickinson) to the tubes. After incubation at 20°C and washing twice with phosphate buffered saline (PBS, containing 0.1 % azide), the samples were analyzed on a FACScan flowcytometer (Becton Dickinson). The number of leukocytes was counted on a Coulter counter.

*Intracytoplasmic cytokine production*

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood by density separation over Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden). Cell cultures were performed in 24-well plates (Nunc, Wiesbaden, Germany). The cells were cultured ($1x10^6$ PBMCs/ml) in Iscove's medium supplemented with 5% heat-inactivated fetal calf serum, penicillin (100 U/ml), streptomycin (100 µg/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). One milliliter of PBMCs was stimulated with a combination of ionomycin (1 µM) (Sigma, St.Louis, MO, USA) and phorbol 12-myrastate 13-acetate (PMA 1 ng/ml) (Sigma) in the presence of monensin (1 ng/ml) (Sigma). As a negative control, cells were incubated without stimulation.

After 5 hours of incubation at 37°C in a humidified atmosphere of 5% carbon dioxide in air, the cells were harvested, washed twice with PBS and fixed with 4% paraformaldehyde for 5 minutes. The cells were then washed again and incubated in PBS/0.1% saponin (Calbiochem, La Jolla, CA, USA)/0.5% bovine serum albumin (BSA, Boseral Organon Technika)/10% human pooled serum (HPS, CLB) (PBS-SBH) for 10 minutes. Cells were resuspended in PBS-SB (without HPS) and incubated for 30 minutes at 4°C with the biotin-labeled anti-cytokine monoclonal antibodies (mAbs) - anti-IL-4 (CLB-5A4) and anti-IFNγ (CLB-MD2) - and IgG1 as a negative control. After washing three times in PBS-SB, the cells were incubated with phyco-erythrin (PE)-labeled streptavidin for 20 minutes at 4°C. The cells were then washed twice in PBS-SB and once in PBS and incubated with FITC-labeled anti-CD3 to isolate the T-lymphocytes. Finally, after 30 minutes of incubation and after washing three times in PBS, the cells were resuspended in 150 µl of PBS and analyzed on a FACScan flow cytometer. Stimulated cells incubated with IgG1 were used as negative controls.

*Whole-blood cell culture*

Whole-blood cell cultures were performed in flat-bottomed micro-titer plates (Nunc, Kamstrup Denmark). Heparinized blood was diluted 1:10 with Iscove's modified Dulbecco's medium (IMDM) supplemented with penicillin (100 U/ml), streptomycin

(100 μg/ml) and 2-mercaptoethanol ($5x10^{-5}$ M). A 200 μl sample of the diluted blood was stimulated with the combination of anti-CD3 (1 μg/ml) and anti-CD28 (5 μg/ml). Incubation of the whole-blood cell cultures was performed at 37°C in a humidified atmosphere of 5% carbon dioxide in air. After 72 hours, the supernatants were harvested and stored at -70°C until cytokine analysis was performed.

*Cytokine assays*

IFNγ. Levels of IFNγ were measured using enzyme-linked immunosorbent assay (ELISA) as described previously with minor alterations.[12] Flat-bottomed 96-well plates were coated with mAb anti-IFNγ (CLB-MD2, 5 μg/ml). Biotinylated sheep anti-IFNγ (CLB-MD1, 2 μg/ml) was used to detect bound IFNγ. The detection limit of this assay was 7 pg/ml and values in healthy control subjects were below 10 pg/ml.

IL-4. Levels of IL-4 were also measured using ELISA, as described previously.[13] Briefly, flat-bottomed micro-titer plates were coated overnight with purified mAb against IL-4 (CLB-4F2) at a concentration of 1 μg/ml. After washing, serial dilutions of IL-4 containing samples were added. Bound IL-4 was detected by biotinylated affinity purified polyclonal sheep anti-IL-4 (5A4). The lower detection level of this assay was 10 pg/ml. The levels in normal healthy control subjects were below 30 pg/ml.

IL-2. The IL-2 ELISA was performed following the same procedure as described above. MAb CLB-B-G5 was coated over night at room temperature (2 μg/ml in PBS, 100 μl per well) on flat-bottom micro-titer plates (Nunc, Maxisorb, Robskilde, Denmark). All subsequent incubations were in 100 μl volumes at room temperature. After three washings with PBS-0.02% (v/v) Tween 20 (PT), an excess of biotinylated purified polyclonal goat anti-IL-2 was added in HPE buffer (High Performance ELISA buffer)-1% normal mouse serum (final concentration 1 μg/ml) at 50 μl per well together with 50 μl of IL-2 containing samples followed by incubation for two hours. Plates were washed (3 x PT) and incubated with streptavidin-poly-horseradish peroxidase (1:10000 diluted, CLB, Amsterdam, The Netherlands) in PBS for 30 minutes, washed and developed with a solution of 100 μg/ml of 3,5,3',5'-tetramethylbenzidine (Merck, Darmstadt, Germany) with 0.003% $H_2O_2$ in 0.11 M sodium acetate, pH 5.5 (100 μl per well). The reaction was stopped by the addition of an equal volume of $H_2SO_4$ to the wells. Plates were read at 450 nm in a Titertek Multiscan reader (Flow Laboratories, Mclean, VA). Human rIL-2 was used as a standard curve; the detection limit was 10 pg/ml. Values in normal healthy control subjects were below 10 pg/ml.

## RESULTS

*Patients*

During perfusion no leakage was detected using radioactive albumin. TNFα was measurable in the systemic circulation from 5 minutes till 3 hours after washout. Maximum TNF-levels were about 2 ng/ml (data not shown).[14] All patients developed a complete tumor response after treatment with ILP.

*Cell-number and -distribution*

The number of T-cells per ml is shown in Figure 1. This number was determined from the percentage of CD3+ cells (measured using a FACScan), and the number of leukocytes was measured on the Coulter counter. The number of T-cells remained constant till the 95 minutes time-point. At 3 hours only 5% of the pre-operative number remained. Next morning the T-cell number had recovered somewhat to about 25% of the pre-operative value. In the perfusate, the T-cell number decreased. At the start of the perfusion, the median T-cell number in the perfusate was $16*10^4$/ml, halfway through it was 57% and at the end of perfusion it was 39% (data not shown).

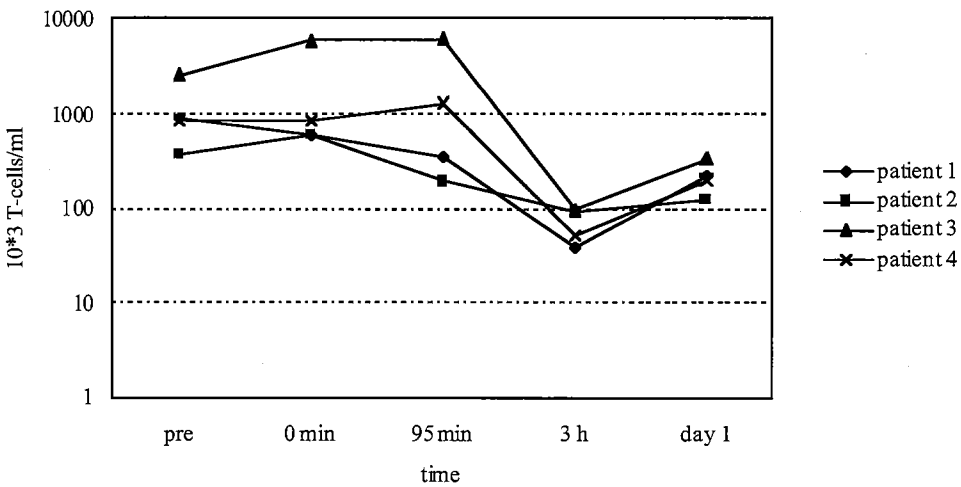


*Figure 1. Absolute number of circulating T cells pre ml in the systemic circulation. Pre, preoperative; 0 min, just before perfusion; 95 min, 95 minutes after the start of perfusion; 3 h, 3 hours after operation; day 1, next morning.*

*Intracytoplasmic cytokine production*

The pre-operative percentage of IFNγ-producing cells was around 20-25% of 5000 counted T-lymphocytes. Three hours after the operation, T-lymphocytes producing IFNγ had declined to 43% (median) of the pre-operative value. On day 1 after the operation, the fraction of IFNγ-producing T-cells was restored to 61% of the initial level (Figure 2). In the perfusate, T-lymphocytes positive for intracytoplasmic IFNγ-production showed a sharp decline from a median of 23% at the start to 12% halfway through perfusion (not measured at the end of perfusion, data not shown).

In all samples IL-4 was not measurable because of very low production, and was not distinguishable from the background signal.

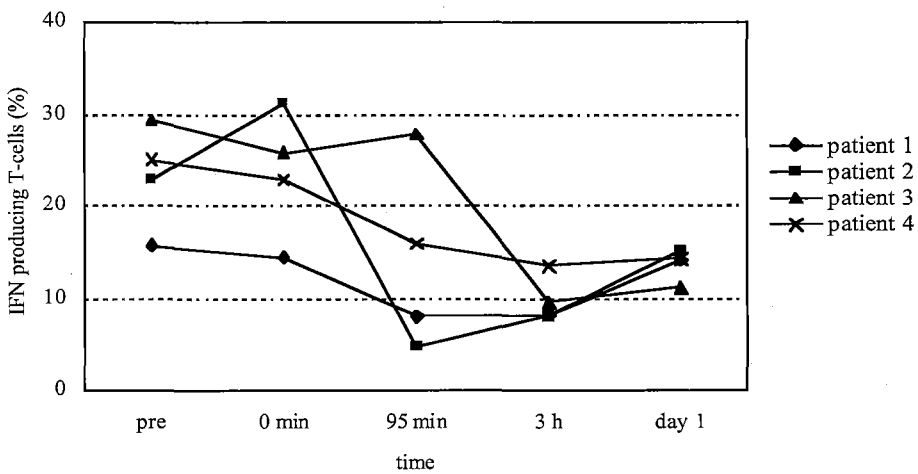


*Figure 2. IFNγ-producing T-cells in the systemic circulation expressed as a percentage of the absolute number of circulating T-cells.*

*Cytokine production in whole-blood culture*

IL-4, IFNγ and IL-2-production in whole-blood culture was expressed in pg per 1000 T-lymphocytes (Figure 3). IFNγ decreased from the start of operation until it reached a level of 1% 3 hours after operation. On day 1 the production was still depressed in three out of four patients.

After the start of the operation, IL-4 production fell to 20% at 95 minutes (5 minutes after the release of the tourniquet). Three hours post-operatively and on day 1, IL-4 was still produced at only a very low level. IL-4 in perfusate was barely measurable.

In addition, production of IL-2 decreased during and after the whole procedure.
In the perfusate, cytokine production was much lower than in the systemic circulation; production of all cytokines showed a decrease during perfusion.

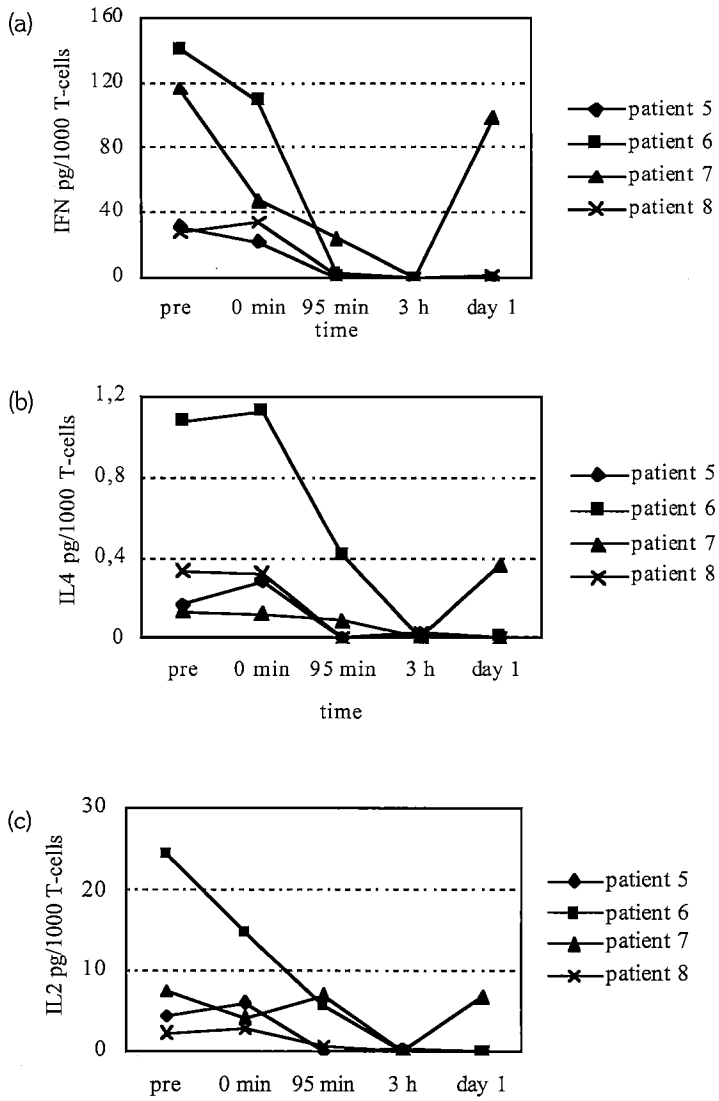


*Figure 3. Production of (a) IFNγ, (b) IL-4 and (c) IL-2 in whole-blood cell culture, expressed in pg per 1000 T-cells in the systemic circulation*

## DISCUSSION

In this study, we have investigated the effect of exposure to TNFα on the Th1/Th2-balance in cancer patients undergoing ILP with TNFα. Cytokine-production was studied at the single-cell level by intracytoplasmic staining after cell stimulation in vitro. Further, it was also determined in the whole-blood cell culture system after specific T-cell stimulation. In our previous study on the effect of TNFα on T-cells, we observed a decrease in the number of T-cells in the circulation during and after ILP.[14] We also studied specific cell populations using cell markers (CD4, CD8). These immunospecific cell markers disappeared, showing the same pattern as CD3+-cells, suggesting that the ratio between the different cell populations remains the same (data not shown). In the present study, to determine whether TNFα would affect the Th1/Th2-balance, we investigated the ability of the remaining T-cells to produce IFNγ and IL-4.

We measured intracellular cytokine-production using the same procedure as described previously.[15-17] In these studies, only 1-2% of the cells were found to be IL-4-positive. Also, in our cancer-patients hardly any IL-4 producing cells could be detected using this method. However, clear changes took place in the IFNγ-producing T-lymphocytes. IFNγ-positive T-cells declined during and after ILP. Next morning the fraction of positive-stained T-lymphocytes was still somewhat depressed. In the perfusate, IFNγ-producing T-cells also disappeared during ILP.

The observations obtained in the intracytoplasmic studies were confirmed by determination of cytokine-production in whole blood cell culture. IFNγ-production after T-cell-stimulation in whole blood cell culture also decreased during and after ILP. At 3 hours and at day 1 after ILP, hardly any IFNγ was measured in the supernatant. In the perfusate, IFNγ-production was completely depressed after 90 minutes. These data were similar to those obtained for IL-2-production; only at 95 minutes was IFNγ lower than IL-2. Next to IFNγ, IL-4 production was also affected by TNFα. From the start of operation until the next morning IL-4 fell, reaching a minimum at day 1. IL-4-production in the perfusate supernatants was measurable only in one patient. It also declined during perfusion.

Our results differ from observations in vitro in which TNFα, added to human T-cells in vitro, stimulated growth and differentiation of T-cells, shown by an increase in TNF- and IL-2-receptor expression on T-cells.[18,19] The concentration of TNFα used (0.2-20 ng/ml) is about the same as the concentration measured in the systemic circulation of our ILP-patients directly after ILP.[14] This observation shows that the situation in vivo is much more complex than in vitro. A probable causal factor could be cortisol, which is secreted in

response to the stress of the operation. Cortisol is known to decrease cytokine-production[20,21] and the number of lymphocytes.[22]

It is clear that TNFα in the ILP-patients has a striking impact on the number of circulating T-cells. TNFα induces adhesion of PBMCs to the endothelium of both tumor-vessels[23] and normal vessels.[24-26] The overall disappearance of T-cells in our patients can, in part, be held responsible for the decrease in IL-4, IL-2 and IFNγ-production in whole-blood cell culture. The infusion of fluids during perfusion causes only a small intravascular dilution because most of the fluids go into the extravascular compartment (operation area and oedema).

In the remaining circulating T-cells, TNFα also had an effect on function, as shown by the intracytoplasmic cytokine production after cell stimulation (Fig.2). The effect of TNFα on T-cells could not be restricted to a specific T-cell-population using the whole-blood cell culture system. IL-4 as well as IFNγ and IL-2 production decreased, suggesting that both Th1- and Th2-cells were affected by TNFα. This could also be concluded from the results measured in the perfusate. If this impaired T-cell function is representative of all T-cells in the systemic circulation, this could be an explanation for the tolerability of high TNFα concentrations after ILP. This tolerability could be caused by a decrease in the synthesis and production of T-cell derived cytokines.

A distinction between the effect of TNFα on cytokine-production and on cell-disappearance could be made. The decrease in cytokine-production preceded the numerical drop in the T-cell population in the systemic circulation: at time-point 95 minutes the number of circulating T-lymphocytes was unchanged (Fig.1), whereas the production of cytokines was already diminished (Fig.3). Three hours after ILP, both cell-number and cell-function were depressed. An explanation for this phenomenon could be that TNFα had an effect on a specific population of cytokine-producing T-helper-cells. This suggestion is sustained by the observation in the intracytoplasmic cytokine-staining procedure: At time-point 95 minutes IFNγ-producing T-cells were reduced, illustrating that some of the cells had disappeared.

In conclusion, we could not show TNFα has a selective effect on Th1- or Th2 cells. Overall, a strong effect was seen on the number of circulating T-cells as well as on the T-cell-function expressed in IFNγ-production and -secretion, IL-4-production and -secretion as well on IL-2-secretion. If the impaired T-cell function in the T-cells remaining in the blood is representative for all T-cells, this could partly explain the tolerability of high TNFα concentrations in the systemic circulation after ILP, perhaps by decreasing the synthesis and production of T-cell-derived cytokines.

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# CHAPTER 4

## SYSTEMIC TOXICITY AND CYTOKINE/ACUTE PHASE PROTEIN LEVELS IN PATIENTS AFTER ISOLATED LIMB PERFUSION WITH TUMOR NECROSIS FACTOR-ALPHA (TNFα) COMPLICATED BY HIGH LEAKAGE

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## ABSTRACT

**Background:** Since the introduction of high-dose tumor necrosis factor-alpha (TNFα) in the setting of isolated limb perfusion (ILP) in the clinic, prevention of leakage to the body of the patient is monitored with great precision for fear of TNF-mediated toxicity. That we observed remarkably little toxicity in patients with and without leakage prompted us to determine patterns of cytokines and acute phase proteins in patients with high leakage and in patients without any leakage.

**Methods:** TNFα, interleukin (IL)-6, IL-8, C-reactive protein (CRP) and secretory phospholipase $A_2$ ($sPLA_2$) were measured at several time-points during and after (until 7 days) ILP in 10 patients with a leakage to the systemic circulation varying in percentage from 12 to 65%. As a control, the same measurements, both in peripheral blood and in perfusate, were performed in 9 patients without systemic leakage.

**Results:** In patients with systemic leakage, levels of TNFα increased during ILP, reaching values to 277 ng/ml. IL-6 and IL-8 peaked 3 hours after ILP with values significantly higher compared with patients without systemic leakage. CRP and $sPLA_2$ peaked at day 1 in both patient groups. $sPLA_2$ with significant higher levels and CRP, in contrast, with lower levels in the leakage-patients.

**Conclusions:** High leakage of TNFα to the systemic circulation caused by a complicated ILP led to a 10-fold to more than 100-fold increased levels of TNFα, IL-6 and IL-8 in comparison with patients without leakage. The increase of the acute phase proteins was limited. Even when high leakage occurs, this procedure should not lead to fatal complications. The most prominent clinical toxicity was hypotension (grade III in 4 patients) which was easily corrected. No pulmonary or renal toxicity was observed in any patient. It is our experience that even in the rare event of significant leakage during a TNFα-based ILP, postoperative toxicity is usually mild and can be easily managed by use of fluid and, in some cases, vasopressors.

## INTRODUCTION

The technique of regional isolated limb perfusion (ILP) with the use of an extracorporeal circuit was pioneered by Creech and coworkers.[1] The advantage of this treatment modality is that high dose of cytostatic drug can be delivered to the tumor-bearing extremity without producing systemic side effects. ILP permits regional cytostatic concentrations 15 to 20 times higher than those reached after systemic administration.[2] The standard drug in this setting is melphalan (L-phenyl-alaninemustard). In patients with multiple melanoma in-transit metastases, an ILP with melphalan results in about 50% of the patients in a complete remission.[3] Addition of tumor necrosis factor-alpha (TNFα) to melphalan has proven most effective in terms of response rate, yielding a 80 to 90% complete response rate, and an overall response of about 100%.[4,5] In locally advanced soft tissue sarcomas the use of TNFα in combination with melphalan has proven remarkably effective in rendering irresectable tumors resectable and thereby preventing amputations.[5,6] The efficacy of TNFα against the drug-resistant soft tissue sarcomas has led to the approval of TNFα by the EMEA (European Medicine Evaluation Agency) for its use in combination with melphalan.[7]

In the ILP system, TNFα is administered in a 10-fold higher dose compared with the maximum tolerable dose in systemic administration. The maximum tolerable dose of TNFα in single-dose intravenous or intramuscular administration is limited by toxicity at 400 $\mu g/m^2$.[8,9] Toxicity consists of fever, hypotension, chills and transient leukopenia. Hardly any tumor response has been reported after systemic administration of TNFα.[9-11] Despite careful precautions, systemic leakage of more than 10% appeared in 10 patients during the last 8 years in our hospital. These patients had a remarkably mild clinical course. To get more insight into the cause of this discrepancy between the high systemic concentration of TNFα and the mild clinical symptoms, we studied cytokine levels and the acute phase response.

## PATIENTS AND METHODS

*Patients*

From the 212 patients who underwent an ILP with TNFα and melphalan in the past 8 years in our hospital, 10 patients were selected because of very high systemic levels of TNFα caused by leakage from the perfusate. These patients were treated because of an irresectable sarcoma (n=6) or melanoma with multiple in transit metastases (n=4). Demographic and treatment characteristics are summarized in table 1. As a control group, we studied 9 comparable patients undergoing ILP without systemic leakage. These patients were all sarcoma-patients, who underwent a 90-minute-long ILP with 3 to 4 mg of TNFα. The mean age was 48 years (range, 21-77 years).

*Table 1. Patient characteristics*

| Patient no. | Age, years | Sex | Diagnosis | Arm/ leg | Duration of perfusion, min | Dose (mg of TNFα | Leak % | Maximum systemic TNFα (ng/ml) |
|---|---|---|---|---|---|---|---|---|
| 1 | 55 | F | Sarcoma | Leg | 90 | 2 | 23 | 169 |
| 2 | 52 | F | Melanoma | Leg | 90 | 4 | 20 | 178 |
| 3 | 66 | M | Sarcoma | Arm | 90 | 3 | 12 | 30 |
| 4 | 61 | F | Sarcoma | Arm | 30 | 3 | 65 | 277 |
| 5 | 71 | F | Melanoma | Leg | 90 | 2 | 24 | 112 |
| 6 | 56 | M | Sarcoma | Leg | 90 | 2 | 15 | 77 |
| 7 | 65 | M | Melanoma | Leg | 90 | 2 | 32 | 108 |
| 8 | 64 | F | Melanoma | Leg | 90 | 4 | 13 | 104 |
| 9 | 83 | M | Sarcoma | Leg | 75 | 4 | 19 | 174 |
| 10 | 55 | F | Sarcoma | Leg | 90 | 4 | 16 | 90 |

*Treatment schedule*

The procedure of ILP has been described previously.[5] In brief, ILP consisted of a 90-minute-long perfusion with 3 to 4 mg of recombinant human TNFα (Boehringer Ingelheim, Alkmaar, The Netherlands) and 10 to 13 mg/liter perfusion tissue of melphalan (Alkeran; Burroughs Wellcome, London, UK) at mild hyperthermia (39 to 40°C). Composition of the perfusate was as follows: priming volume of 700 to 850 ml consisted of 400 to 500 ml of blood (50% red blood cells/50% plasma), 200 to 400 ml

of 5% dextran-40 in glucose 5% (Isodex, Pharmacia, Uppsala, Sweden), 10 to 30 ml of 8.4% sodium bicarbonate and 0.5 ml of 2500 to 5000 IU heparin. TNFα was injected as a bolus into the arterial line provided limb tissue temperature was >38°C. Melphalan was administered 30 minutes later at limb temperatures between 39 to 40°C. At the end of perfusion, the limb was washed with at least 2 liters of 6% dextran-70 (Macrodex, Pharmacia). During and after ILP, vital signs of the patients, including body temperature, heart rate, blood pressure and fluid balance were recorded. Toxicity was registered according to the World Health Organization criteria.[12]

*Leakage monitoring*

During ILP, there was a dynamic balance between two pressure compartments, the systemic vasculature and the isolated circuit, which could be influenced by adjusting the systemic blood pressure and/or the extracorporeal flow rate. Throughout the perfusion period, any potential leakage of the drugs was monitored by using a radioactive tracer. A small calibration dose of human serum albumin radiolabeled with iodine 131 or technetium 99m was injected into the systemic circulation and a 10-fold higher dose of the same isotope into the isolated extremity. Continuous monitoring was performed with a precordial scintillation probe. Systemic leakage was expressed quantitatively as a percentage such that 100% leakage represented a homogeneous distribution of the isotope in the body.

*Blood sampling procedure*

Venous blood samples were collected at several time-points, i.e., the day before ILP, just before administration of TNFα in the perfusate, halfway perfusion and just before release of the tourniquet (after completion of the washout procedure at the end of the perfusion). Then after ILP, 5, 10, 30 minutes and 3 and 7 hours after release of the tourniquet and once a day until 7 days after ILP. Samples from the perfusate were obtained at the following times: 0 (just before administration of TNFα) and 10, 30, 60 and 90 minutes after administration of TNFα. Blood samples were immediately centrifuged; plasma was collected and stored at -70°C until tested.

*Assays for cytokine and acute phase protein analysis*

Cytokine and acute phase protein levels were measured by using enzyme-linked immunosorbent assay (ELISA). Used antibodies were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). For

measuring TNFα, as described previously,[13] flat-bottomed microtiter plates (Nunc, Kamstrup, Denmark) were coated overnight with purified monoclonal antibody (mAb) against TNFα (CLB-TNF/7). After washing serial dilutions of TNF-containing samples were added. Bound TNFα was detected by biotinylated sheep anti-TNFα. The detection limit of the assay was 5 pg/ml. Healthy controls were at less than 5 pg/ml.
The IL-6-specific ELISA has been described previously.[14] A coat of CLB-IL-6/16 was applied overnight, and bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. The lower detection limit was 1 pg/ml and normal healthy control subjects were at less than 10 pg/ml.
For IL-8, a coat of CLB-IL-8/1 mAb was applied overnight and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8. The lower detection limit of this assay was 8 pg/ml. Normal values were at less than 20 pg/ml.[15]
C-reactive protein (CRP) levels were measured by a sandwich ELISA, using polyclonal rabbit anti-human CRP Abs as catching Abs and biotinylated mAb anti-CRP (CLB anti-CRP-2) as a detecting Ab. Results were referred to a standard (Behringwerke AG, Marburg, Germany) and expressed in mg/l. The detection limit was 10 ng/liter.[16]
The ELISA used for measuring secretory phospholipase $A_2$ ($sPLA_2$) has been described before.[17] Two different mAbs against human $sPLA_2$ were used as coating and catching antibodies respectively. The lower limit of detection was 0.1 ng/ml. Normal healthy volunteers were at less 5 ng/ml.

*Statistics*
Median values are expressed with range. Comparison between the cytokine and acute phase protein levels in the two groups (with and without leakage) were made by the Mann-Whitney U test. Values of $p \leq 0.05$ were considered to be statistically significant.

## RESULTS

*Systemic toxicity*
Ten patients with a systemic leakage percentage of more than 10% were entered this study. Leakage varied from 12% to 65% (mean 24%, table 1). Because of expected toxicity all patients are well monitored at our intensive care unit post-operatively. Systemic toxicity is summarized in table 2.

*Table 2. Systemic toxicity following ILP in the 10 patients with leakage to the systemic circulation compared to 9 patients without leakage*

| | Toxicity (WHO grade) | | | |
|---|---|---|---|---|
| | Grade 0-I | Grade II | Grade III | Grade IV |
| Leakage | | | | |
| Fever | 2 | 8 | 0 | 0 |
| Hypotension | 3 | 3 | 4 | 0 |
| Leukocytes [a] | 8 | 0 | 1 | 1 |
| Platelets [b] | 6 | 3 | 0 | 1 |
| Bilirubin [c] | 3 | 3 | 4 | 0 |
| ALAT/ASAT [d] | 7 | 3 | 0 | 0 |
| Nausea | 8 | 2 | 0 | 0 |
| | | | | |
| Non-leakage | Grade 0-I | Grade II | Grade III | Grade IV |
| Fever | 6 | 3 | 0 | 0 |
| Hypotension | 9 | 0 | 0 | 0 |
| Leukocytes | 9 | 0 | 0 | 0 |
| Platelets | 9 | 0 | 0 | 0 |
| Bilirubin | 9 | 0 | 0 | 0 |
| ASAT/ALAT | 9 | 0 | 0 | 0 |
| Nausea | 8 | 1 | 0 | 0 |

WHO, World Health Organization; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase.

[a] gr. III: 0-1.9 x $10^9$/liter; gr.IV: < 1.0 x $10^9$/liter

[b] gr. II: 50-74 x $10^9$/liter; gr.IV: < 25 x $10^9$/liter

[c] gr. II: 2.6-5 x N; gr. III: 5.1-10 x N (N = upper limit of normal value)

[d] gr. II: 2.6-5 x N

During ILP, blood pressure and pulse rate remained stable with adequate fluid management. The body-temperature did not increase above 38°C. After ILP, all patients received indomethacin to suppress flu-like symptoms. Eight of 10 patients developed fever grade II (38 to 40°C) within a few hours after ILP (mean maximal temperature 38.9°C). In the patients without detectable leakage the mean maximal temperature was 38.1°C. In 4 leakage-patients, the heart rate increased to more than 110 beats per

minute (range 120-132 beats per minute). Four patients had a hypotension, which was not quickly restored to normal values by fluid administration alone, and required additional treatment with dopamine (3-6 μg/kg/min) during 2 to 3 days. From the start of surgery, a mean of 8 liters was administered to the leakage-patients during the first 16 hours versus 5 liters for non-leakage patients. Leukopenia and thrombocytopenia was absent or mild. Grade IV leukopenia and thrombocytopenia in one patient was induced by leakage of melphalan. No transfusion was required. Patients without leakage did not develop hematological toxicity. All leakage-patients had a hyperbilirubinemia after 2 days and the transaminases increased (grade I-II). In the no-leakage group, only 1 patient had a mild hyperbilirubinemia. No pulmonary or renal toxicity was observed in any patient.

**Plasma cytokine and acute phase protein levels**

In Figure 1, median values with range were represented for the cytokines TNFα and IL-6 and the acute phase proteins (APPs) CRP and $sPLA_2$. Median peak levels of all measured cytokines and APPs in both patient groups, depicted in table 3, were significantly different (p-values in Table 3). Because we know the curves of IL-8 and $sPLA_2$ from previous published experiments[18, 19] we restricted the determinations to the pre-operative and the maximum level time points.

In the leakage group, very high circulating concentrations of TNFα are found *during* perfusion (at – 45 minutes) in contrast to the non-detectable TNFα-levels in patients without leakage. Plateau circulating concentrations are measured at the end of the perfusion, lasting up to 30 minutes after ILP. The small amount of TNFα that remains in the limb after the washout procedure does not increase the colossal systemic levels any further in these patients in contrast to what is observed in patients without leakage. There we observed a brief peak of systemic levels more than 100-fold less than in leakage patients. Moreover, the peak occurs typically at 5-10 minutes after ILP and represents the TNFα that was left behind in the limb after the washout. TNF-levels decreased already after 30 minutes, because rapid clearance of this cytokine with a short half-life time of 17 minutes is operational. Thus, in leakage-patients very high TNF-concentrations are present for about 4 hours, whereas a very short peak of 20 to 30 minutes of "moderate" increased TNF-levels is present in leakage-free patients. There was a strict correlation between the degree of leakage estimated by isotope monitoring, the (adjusted) dose of TNFα and the measured maximum systemic levels of TNFα, depicted in table 1. IL-6 increased already during perfusion in leakage-patients, immediately induced by TNFα. In the non-leak patients IL-6 increased 5 to 10 minutes after ILP, i.e. after TNFα

from the washed out limb appeared in the systemic circulation. Maximum values of IL-6 were reached 3 hours after ILP. IL-8 showed the same pattern as IL-6 (data not shown). In the control group, values of IL-6 and IL-8 were 10 to 60 times lower than in the leakage-patients.
The APP CRP was increased from 3 hours after ILP until more than 7 days after ILP. Peak levels occurred at day 1. The CRP curve in patients without leakage was comparable, but the peak value was higher. Levels of $sPLA_2$ were very different for each patient. However, the pattern was consistent, with the start of increase at 3 hours after ILP and the peak at day 1. Levels were still increased after 7 days. Levels in the non-leakage patients were factor 6 lower.

**Levels of cytokines and acute phase proteins in perfusate**

No significant difference was observed in perfusate-levels of cytokines and APPs in patients who underwent ILP with systemic leakage and without leakage. From the ILP-patients with systemic leakage, only 5 series of perfusate samples were available. Curves are presented in figure 2. TNF levels remained stable around 7.5 μg/ml. IL-6 increased after 10 minutes of perfusion, to 4.3 ng/ml at the end of perfusion. IL-8 increased from 65 to 1600 pg/ml during perfusion. CRP did not change during perfusion, with values hardly detectable or at less than the detection limit. In 3 of 5 patients $sPLA_2$ increased during perfusion; the median value at the end of perfusion was 14 ng/ml and the range 8.5 to 266 ng/ml.

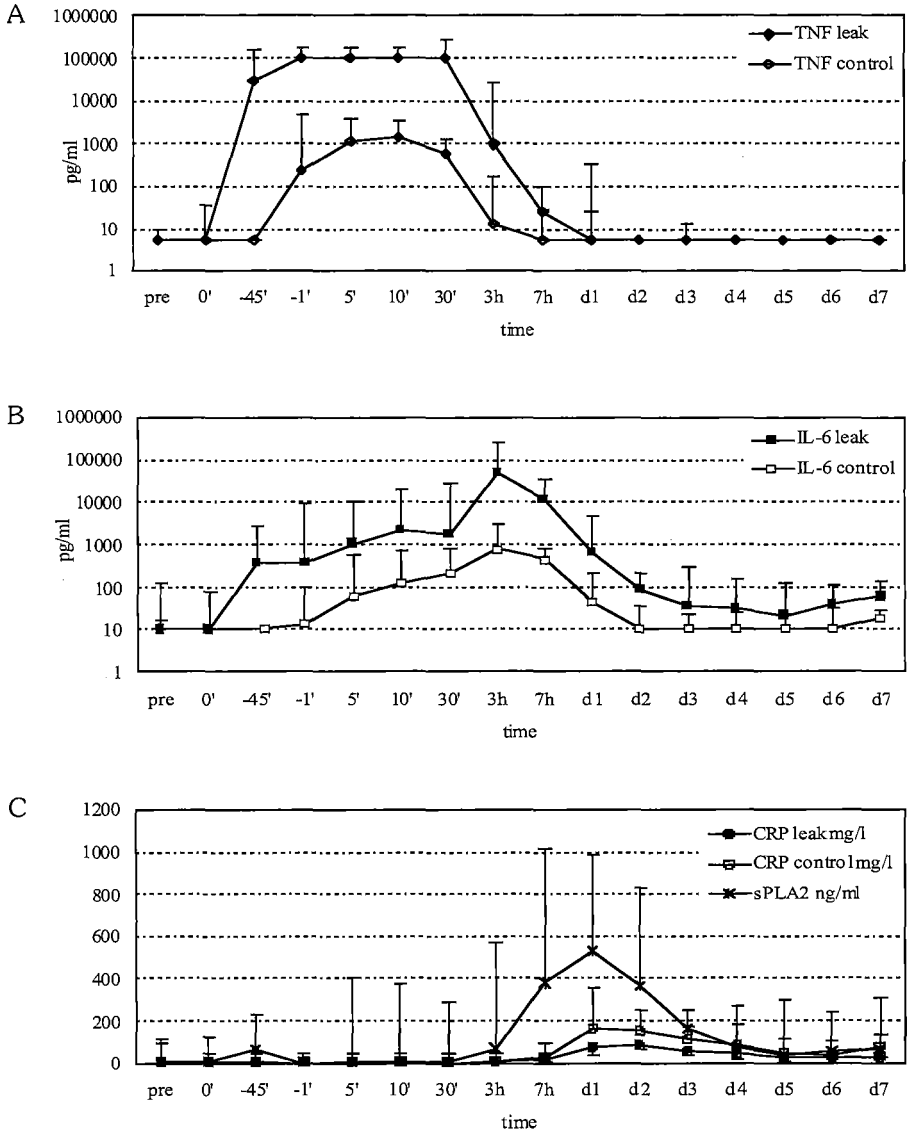


*Figure 1. Median (with range) of TNF (A) and IL-6 (B) and CRP and $sPLA_2$ (C) in plasma from 10 patients who underwent an ILP complicated by high leakage (> 12%) to the systemic blood circulation. For TNF (◇; A), IL-6 (□;B) and CRP (O; C), the curves of the non-leakage group are also depicted. Time-points were as follows: pre, pre-operative; 0', just before perfusion; -45', halfway perfusion; -1', just before release of the tourniquet; 5' (10' and 30'), 5 (10 and 30) minutes after release of the tourniquet; 3h and 7h, 3 and 7 hours after ILP; d1 to d7, number of days after ILP.*

*Table 3. Median and range of peak-levels of cytokines and acute phase proteins in 10 patients undergoing ILP with more than 10% leakage compared with 9 patients without leakage in the systemic circulation*

| Cytokine/acute phase protein | Time-point | Leakage-patients, median (range) | No leakage, median (range) | p-value |
|---|---|---|---|---|
| TNFα, ng/ml | 10 min after ILP | 108 (26-277) | 1.4 (0.3-3.4) | p<0.001 |
| IL-6, ng/ml | 3 hours after ILP | 49 (13-257) | 0.8 (0.3-3.3) | p<0.001 |
| IL-8, ng/ml | 3 hours after ILP | 14 (1.3-49) | 0.2 (0.01-1.7) | p<0.001 |
| CRP, mg/l | Day 1 | 76 (34-419) | 166 (93-350) | p<0.01 |
| $SPLA_2$, ng/ml | Day 1 | 568 (123-986) | 84 (20-390) | p<0.01 |

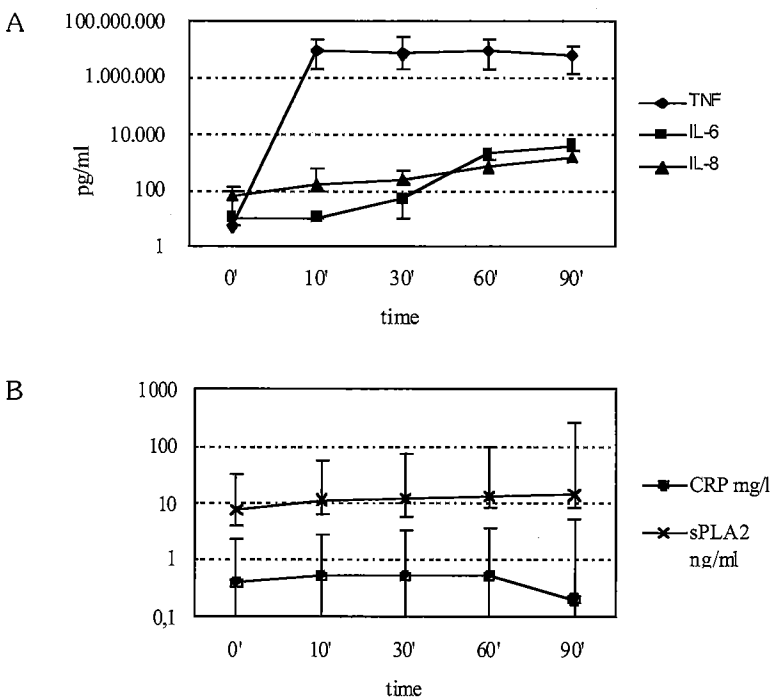


*Figure 2. Median (with range) of cytokine (A) and acute phase protein (B) profile in perfusate from 5 patients who underwent an ILP complicated by high leakage (> 12%) to the systemic blood circulation. Time-points were as follows: 0', just before administration of TNFα in the perfusate; 10' (30' and 60'), 10 (30 and 60) minutes after start of perfusion; 90', end of perfusion, just before release of the tourniquet.*

## DISCUSSION

The aim of our study was to quantify cytokine levels and acute phase response in patients who underwent an ILP with high dose TNFα complicated by high leakage, compared with the same variables in patients without leakage, and correlate findings with clinical toxicity. In this study, we measured TNFα plasma-levels up to 277 ng/ml. Nevertheless, most of the patients needed only extra intravenous fluid administration. Systemic levels of this magnitude have been described before in the same setting [20,21] and once after a 30-min intravenous infusion of recombinant TNFα.[22]

In our patients the necessity for dopamine administration was not related to the highest levels of TNFα in the systemic circulation. This finding is in accordance with previous studies in which no correlation between maximum TNFα concentrations in the peripheral blood of an individual and the side-effects could be found,[20,23] which indicates that patients vary in their sensitivity to TNFα.

Systemic toxicity seems to be determined by the duration of exposure to high levels of TNFα. Our data demonstrate this clearly, with levels of 1000 to more than 100,000 pg/ml for 4 hours in high leakage patients, and only "moderate" levels (~1000 pg/ml for 20 minutes) in non-leakage patients. In non-leakage patients, 20 minutes of "moderate" TNF-levels were not enough to cause hypotension. This is in accordance with the findings reported previously in this journal by Vrouenraets et al., who described minimal toxicity after leakage-controlled ILP in 20 patients.[24] The IL-6 curves demonstrate the effect of prolonged exposure to high TNF levels even more pungently. Even at 24 hours after ILP, IL-6 levels are still higher in the leakage-patients than the peak IL-6 levels observed in ILP-patients without leakage. IL-6 levels remained elevated for at least 3 whole days.

In the 10 patients with high leakage, 4 hours of exposure to very high levels of TNFα resulted in 3 patients with grade II and in 4 patients with grade III hypotension. Four patients required dopamine support temporarily with good response. In phase I-II studies on the systemic administration of TNFα, dose-limiting toxicity was observed at TNF-concentrations similar to those observed in our 10 patients described here. For instance, Schaadt et al. reported dose-limiting hypotension in 32% of the patients after administration of 650 μg/m2 intravenously, resulting in a systemic TNF peak concentration of approximately 270 ng/ml. Moreover, grade II hepatotoxicity was observed in 80% of the patients. This is quite different from the relative lack of toxicity observed in our 10 ILP-patients who had similar systemic TNF peak concentrations. In other studies, hypotension was dose-limiting at lower doses, with serum TNF levels of 10 ng/ml. [8,9]

In comparison with septic shock, the duration of exposure to elevated levels of TNFα in the leakage-patients is relatively short. The prolonged exposure in septic shock, despite of concentrations many times lower than the short peak levels after ILP, results in the typically unresponsiveness of the hypotension to fluid challenge in the septic shock patients.[25-28] Adequate diuresis plays a key-role to keep the period of high circulating TNF-levels as short as possible, to prevent a septic shock-like state in perfusion patients. That patients are well hydrated at the time of exposure to TNF, and that their blood pressure is optimally maintained by fluid challenge, and only if necessary also by the use of dopamine, prevents septic shock-like situation. This explains why these patients have little toxicity in view of the very high circulating TNF-levels. It is a fundamental difference, with often poorly hydrated patients with metastatic cancer who received intravenous TNFα in phase I-II studies in the past. Moreover, septic patients are infected and have significant levels of endotoxin, which has been shown to be synergistic with TNFα for toxicity (in rats).[29,30] In addition Feelders et al. have shown in patients who underwent ILP, cortisol is already increased before the TNF-peak as a result of surgery and anesthesia.[31] The cortisol response may have a down-regulatory effect on TNFα.

The increased TNFα-levels in the patients with systemic leakage were followed by significantly higher levels of IL-6 and IL-8. This is in accordance with previous studies.[18,20,32] In our study, we also determined CRP and $sPLA_2$ as parameters of the acute phase response. $sPLA_2$ levels were more increased in the leakage-patients; CRP, in contrast, had significantly lower levels in these patients at the time points of maximum values. Lower levels of CRP than expected were also observed in patients who underwent an isolated hepatic perfusion.[33] That CRP had even lower levels in leakage-patients could be ascribed to a higher expenditure of CRP in the neutralization of the effects of exposure to higher levels of TNFα. [34-36]

In conclusion, ILP complicated by high leakage to the systemic circulation, resulted in high systemic levels of TNFα up to 277 ng/ml. IL-6 and IL-8 followed with significantly higher levels compared with values measured in patients without leakage. The pattern of the APPs CRP and $sPLA_2$ resembled each other, except that CRP levels had significantly lower maximum levels in leakage-patients compared with patients without leakage. Overall, the patients with high systemic leakage had a marked mild clinical course. It is our experience that, even in patients with very high leakage, life-threatening reactions have not occurred and that temporary hypotension can be easily dealt with by fluid challenge and sometimes by temporary vasopressor support. Our observations support the need for further study of the potential use of TNFα systemically.

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# CHAPTER 5

## INTRAPLEURAL ADMINISTRATION OF TUMOR NECROSIS FACTOR-ALPHA (TNFα) IN PATIENTS WITH MESOTHELIOMA: CYTOKINE PATTERNS AND ACUTE PHASE PROTEIN RESPONSE

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## ABSTRACT

**Background:** Tumor necrosis factor- alpha (TNFα) has been found to be very effective in the isolated limb perfusion setting for advanced extremity tumors. In a phase I study of intrapleural administration of TNFα 5 patients were followed for inflammatory response patterns.

**Patients and methods:** Malignant mesothelioma patients were treated with repeated intrapleural administration of 0.1-0.2 mg recombinant TNFα. Samples of serum and pleural fluid were taken at different time-points before and after TNFα-administration. Levels of TNFα, interleukin-6 (IL-6), interleukin-8 (IL-8), C-reactive protein (CRP) and secretory phospholipase $A_2$ ($sPLA_2$) were measured using enzyme-linked immunosorbent assays (ELISAs). Alpha 1-acid glycoprotein (α1-AG) was measured by nephelometry.

**Results:** In pleural fluid TNFα and IL-8 reached peak levels, up to 50-700 ng/ml and 6-60 ng/ml, respectively, 24 hours after administration of TNFα. IL-6 (peak levels up to 250 ng/ml) and $sPLA_2$ peaked after 48 hours. A slower and less dramatic pattern was observed for the levels of CRP and α1-AG. In serum no detectable levels TNFα and IL-8 were observed, whereas serum levels of IL-6, $sPLA_2$ and CRP showed a clear increase after intrapleural administration of TNFα. Cytokines and acute phase proteins showed the same pattern during subsequent cycles even up to 12 cycles. Tumor regression was not observed.

**Conclusions:** In the setting of a phase I study of repetitive intrapleural administration of TNFα in mesothelioma patients, we studied the characteristics of the inflammatory response. Intrapleural administration was followed by a clear inflammatory response locoregionally. In spite of TNFα peak levels as high as 700 ng/ml systemic levels were never detectable. The secondary cytokine response led to very high intrapleural IL-6 and IL-8 levels. Systemically, IL-8 levels were never detectable whereas high IL-6 levels were induced systemically initially, with a decreased response to each intrapleural TNFα administration over time. The acute phase response in contrast remained remarkably constant throughout the course of repeated intrapleural administrations of TNFα. Intrapleural administration of TNFα is well tolerated, but associated with inconsistent and rather moderate impact on production of pleural fluid. This can be achieved by other simpler and cheaper treatment, thus we see no justification for further studies.

## INTRODUCTION

Malignant mesothelioma is a tumor associated with exposure to asbestos.[1] Recent studies have indicated that the incidence of malignant mesothelioma with its long time between exposure and presentation of the disease will continue to rise in the future.[2]
Malignant mesothelioma is a notoriously refractory tumor to all current treatments. Neither surgery[3] nor radiotherapy[4,5] results in an increased survival. The median survival of mesothelioma is about 10 months.[4,6,7] Therefore various alternative approaches have been tested.[8] Pleural mesothelioma tends to stay locoregionally throughout most of its natural course.[9] This biological behaviour makes this disease amenable to locoregional administration of cytostatic agents or biologic agents such as interferon-gamma (IFNγ)[10], interferon-alpha[11], IL-2.[12-14] With intrapleural administration of IFNγ or IL-2 antitumor responses have been recorded and it was speculated that TNFα might be implicated in these antitumor effects. Thus we decided to explore intrapleural administration of TNFα, a cytokine that had failed systemic administration because of excessive toxicity.[15,16] Therefore the clinical use of TNFα is restricted to locoregional application. It is already very successfully used in the treatment of irresectable sarcoma and melanoma.[17,18]
There are very few reports on intracavitary TNFα in humans. TNF has been administered intraperitoneally in patients with advanced peritoneal carcinomatosis.[19,20] Regional toxicity (abdominal pain) instead of systemic toxicity was the dose-limiting factor in this setting. Intrapleural administration was studied in patients with malignant pleural effusion by Karck et al.[21] Treatment with up to 200 $\mu g/m^2$ weekly, led in 3 out of 6 patients to disappearance of effusion. Because of this preclinical and clinical data, we decided to perform a phase I study in patients with stage I-IIA malignant pleural mesothelioma with TNFα to study the clinical effect on pleural mesothelioma and evaluate the inflammatory response after this mode of administration.
We have shown previously that leakage of TNFα during isolated limb perfusion (ILP) caused an acute phase response.[22] This was demonstrated by an increase of IL-6 directly after ILP until 2 days thereafter, followed by increase of CRP, α1-AG and α1-antitrypsin after 1 day, and decrease of negative acute phase proteins albumin and transferrin during ILP till 6 hours after ILP.
In the literature it is questioned whether the acute phase protein response (APR) could be downregulated by a repeated stimulus.[23] Clinical studies have shown that in a number of chronic inflammatory diseases the APR is less than would be expected for the activity of inflammation.[24-26] In our patients a repeated stimulus is mimicked: at regular

time intervals of 2 weeks, TNFα is administered. This enables us to investigate whether *repeated* administration of TNFα is still able to provoke an acute phase response.

## PATIENTS AND METHODS

*Patients*

Five patients with pleural mesothelioma in a phase I study were sampled for evaluation of biologic response patterns. Demographical and clinical characteristics are summarized in Table 1. Staging and diagnosis of mesothelioma was based on computerized tomographic (CT) scan of the chest, thoracoscopic findings and histological examination of biopsy samples. According to Butchart's staging system[27] stage I is defined as tumor confined within the capsule of the parietal pleura, i.e. involving only ipsilateral pleura, lung, diaphragm and external surface of the pericardium within pleural reflection. Stage IIA is defined as mesothelioma invading chest wall or mediastinal tissues with or without lymphnode involvement ipsilaterally inside the chest.

Eligibility criteria required histologically confirmed pleural mesothelioma stage I-IIA, sufficient pleural effusion to insert an intrapleural catheter, no signs of loculation on the CT scan, no prior chemo-, radio- or immunotherapy, age < 76 years, Karnofsky performance status ≥ 80%, no cardiovascular disease, a white blood cell count ≥ $4.0 \times 10^9$/l, platelets ≥ $100 \times 10^9$/l, serum bilirubin and creatinine levels within the institution's normal range, no active infection, no use of corticosteroids and obtained informed consent. The protocol was approved by the hospital's ethical committee.

*Table 1. Patient characteristics and treatment response*

| Patientno. | Age | Sex | Stage | Treatment after 6x | Response after 12x | Response |
|---|---|---|---|---|---|---|
| 1 | 63 | M | I | 1 x 0.1 mg | - | - |
| 2 | 65 | M | I | 12 x 0.1 mg | SD | PD |
| 3 | 63 | M | IIA | 18 x 0.2 mg | SD | SD-PD |
| 4 | 65 | M | I | 6 x 0.1 mg | PD | - |
| 5 | 57 | M | I | 6 x 0.1 mg | PD | - |

*Treatment schedule*

About 2 weeks before the first administration of TNFα a Port-a-cath system was surgically inserted under general anaesthesia. The correct intrapleural position was examined radiographically and a technetium-99m colloid scan was made to evaluate the distribution of pleural fluid throughout the pleural cavity.

Recombinant human TNFα (Boehringer Ingelheim, Germany) was administered as an intrapleural infusion, repeated every 14 days. Four patients were treated with a dose of 0.1 mg, one patient with 0.2 mg.

*Tumor response evaluation*

Response was evaluated after every 6 cycles using CT scan of the chest. Tumor response and toxicity were assessed according to the criteria of the World Health Organization (1979).[28] In case of measurable disease, complete response (CR) was defined as the disappearance of all known disease for at least 4 weeks; partial response (PR) as a decrease of >50% in tumor size for at least 4 weeks; stable disease (SD) as a decrease of <50% in tumor size. Progressive disease (PD) was defined as an increase of >25% in the diameter of any lesion or the appearance of a new lesion.

*Immunomonitoring and cytokine- and acute phase protein assays*

Both serum samples and pleural fluid were collected at 4 time-points during each cycle: 24 hours before administration of TNFα, 24 hours and 48 hours after administration and at day 8. All samples were cryopreserved until testing.

Levels of TNFα, IL-6, IL-8, CRP and $sPLA_2$ were measured using enzyme-linked immunosorbent assays (ELISA). Used antibodies were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands).

For measuring TNFα, as described previously[29], flat-bottomed microtiter plates (Nunc, Kamstrup, Denmark) were coated overnight with purified monoclonal antibody (mAb) against TNFα (CLB-TNF/7). After washing, serial dilutions of TNF-containing samples were added. Bound TNFα was detected by biotinylated sheep anti-TNFα. The detection limit of the assay was 5 pg/ml. Healthy controls were below 5 pg/ml.

The IL-6 specific ELISA was described previously.[30] A coat of CLB-IL-6/16 was applied overnight and bound IL-6 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-6. Lower detection limit was 1 pg/ml and normal healthy control subjects were below 10 pg/ml.

For IL-8 a coat of CLB-IL-8/1 monoclonal antibody (mAb) was applied overnight and bound IL-8 was detected by biotinylated affinity-purified polyclonal sheep anti-IL-8. The lower detection limit of this assay was 8 pg/ml. Normal values were below 20 pg/ml.[31]

CRP levels were measured by a sandwich ELISA using polyclonal rabbit antihuman CRP Abs as catching Abs and biotinylated mAb anti-CRP (CLB anti-CRP-2) as a detecting Ab.[32] Results were referred to a standard (Behringwerke AG, Marburg, Germany) and

expressed in mg/l. Detection limit was 10 ng/l.
The ELISA used for measuring $sPLA_2$ was described before.[33] Two different mAbs against human $sPLA_2$ were used as coating and catching Abs respectively. The lower limit of detection was 0.1 ng/ml. Normal healthy volunteers were below 5 ng/ml.
Levels of α1-acid glycoprotein were measured by means of a nephelometric assay. Antisera were obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands). Normal values were 400-900 mg/l.

## RESULTS

*Cytokine and acute phase protein levels in pleural fluid*

Peak-levels (median with range) of cytokines and acute phase proteins of all patients are represented in Table 2. In Figure 1 measurements of the first 6 cycles of patient 2 are depicted, the results of the other patients were comparable. After TNF-administration the concentration of TNFα increased in 24 hours to levels in the range of 50 to 100 ng/ml. After 48 hours still some TNFα was measurable (150-500 pg/ml). No TNFα was measurable after 8 and 14 days. Before the first administration of TNFα IL-6 was increased in all patients, values varying from 1200 to 80,000 pg/ml. It rose sharply to 100 ng/ml after 24 hours and 250 ng/ml after 48 hours. After 8 days it declined to 30-80 ng/ml, after 14 days levels were in the range of 15-33 ng/ml. IL-8 started at 180-260 pg/ml. It increased sharply during in the first 24 hours to 2-12 ng/ml, then it decreased to 0.7-1.0 ng/ml at 48 hours and about 200 pg/ml after 8 days. After 14 days levels were around 150 pg/ml. Levels of CRP increased during the first cycle from 3 mg/l at start to 60 mg/l after 8 days. Thereafter values remained constant around 60 mg/l. $sPLA_2$ increased during 48 hours from around 5 ng/ml (3-9 ng/ml after 24 hours) to 20-30 ng/ml. It decreased slowly over the following 2 weeks. Levels of α1-AG remained constant around 1300 mg/l after a slow rise from 700 mg/l. The production of cytokines demonstrated the same pattern even after cycle 12 in patient 2.

*Serum-cytokine and acute phase protein levels*

In serum (Figure 2, Table 2) no TNFα and no IL-8 was measurable in any patient. IL-6 could not be detected in two patients but increased sharply in the other three, 24 hours after TNF-administration; values were in the range of 125-275 pg/ml. At 48 hours it was still measurable in the first 4 cycles. In the course of treatment, IL-6 levels gradually decreased. Before the first administration of TNFα, CRP-levels in serum were elevated in all patients (31-300 mg/l). CRP-levels increased slowly from around 100 mg/l after 24 hours to 500 mg/l after 48 hours. After 8 days CRP was still measurable with values in the range of 100 mg/l. After 14 days values were around 40 mg/l. $sPLA_2$ was increased after 24 and 48 hours to about 30 ng/ml and 130 ng/ml, respectively. After a few cycles, $sPLA_2$ was also measurable after 8 days: about 7 ng/ml. Levels of α1-AG were elevated before administration of TNFα (1200-2000 mg/l), it increased after 24 hours after administration to about 2500 mg/l at day 8. After 14 days it was around 2000 mg/l.

*Table 2. Peak values (median, range) of measured cytokines and acute phase proteins in 5 patients*

| Cytokine/ Acute phase | | Serum | | | Pleural fluid | | |
|---|---|---|---|---|---|---|---|
| Protein | Pat | Time | Median | Range | Time | | Median Range |
| TNFα pg/ml | 1* | no peak | <5 | | 24h | 30000 | |
| | 2 | no peak | <5 | | 24h | 51500 | 48000-118000 |
| | 3 | no peak | <5 | | 24h | 678000 | 190000-1755000 |
| | 4 | no peak | <5 | | ND | | |
| | 5 | no peak | <5 | | 24h | 2156000 | 218000-4395000 |
| IL-6 pg/ml | 1 | 24h | 160 | | day 8 | 179000 | |
| | 2 | 24h | 173 | 67-279 | 3x48h,3x24h | 248000 | 187500-284000 |
| | 3 | no peak | <1 | | no peak | | 18000-142000 |
| | 4 | no peak | <1 | | ND | | |
| | 5# | 24h | 113 | 91-135 | 24h | 286000 | 148500-393000 |
| IL-8 pg/ml | 1 | no peak | <8 | | 24h | 6300 | |
| | 2 | no peak | <8 | | 24h | 6000 | 2200-12400 |
| | 3 | no peak | <8 | | 24h | 64400 | 51000-71000 |
| | 4 | no peak | <8 | | ND | | |
| | 5 | no peak | <8 | | 24h | 186500 | 3500-261000 |
| CRP mg/l | 1 | 48h | 499 | | day 14 | 83 | |
| | 2 | 48h | 501 | 385-618 | no peak | | 3-69 |
| | 3 | no peak | | 70-223 | no peak | | 31-81 |
| | 4 | 48h | 276 | 216-446 | ND | | |
| | 5 | 48h (2x24h) | 409 | 327-875 | day 8 | 88 | 71-198 |
| SPLA2 ng/ml | 1 | 48h | 67 | | 48h | 23 | |
| | 2 | 48h | 131 | 59-142 | 48h | 25 | 16-31 |
| | 3 | 48h | 11 | 9-19 | no peak | | 0.1-7 |
| | 4 | 48h | 47 | 28-78 | ND | | |
| | 5 | 48h (3x24h) | 87 | 65-174 | 48h | 21 | 17-64 |
| a1-AG mg/l | 1 | day 8 | 2420 | | ND | | |
| | 2 | 3xd8,3x48h | 2350 | 2090-2530 | no peak | | 740-1560 |
| | 3 | no peak | | 1800-2610 | ND | | |
| | 4 | 48h | 2440 | 2320-2630 | ND | | |
| | 5 | no peak | | 2100-2540 | no peak | | 740-1620 |

*Only 1 cycle

#IL-6 was measurable only during the first two cycles, thereafter it was under detection limit. ND: not measured

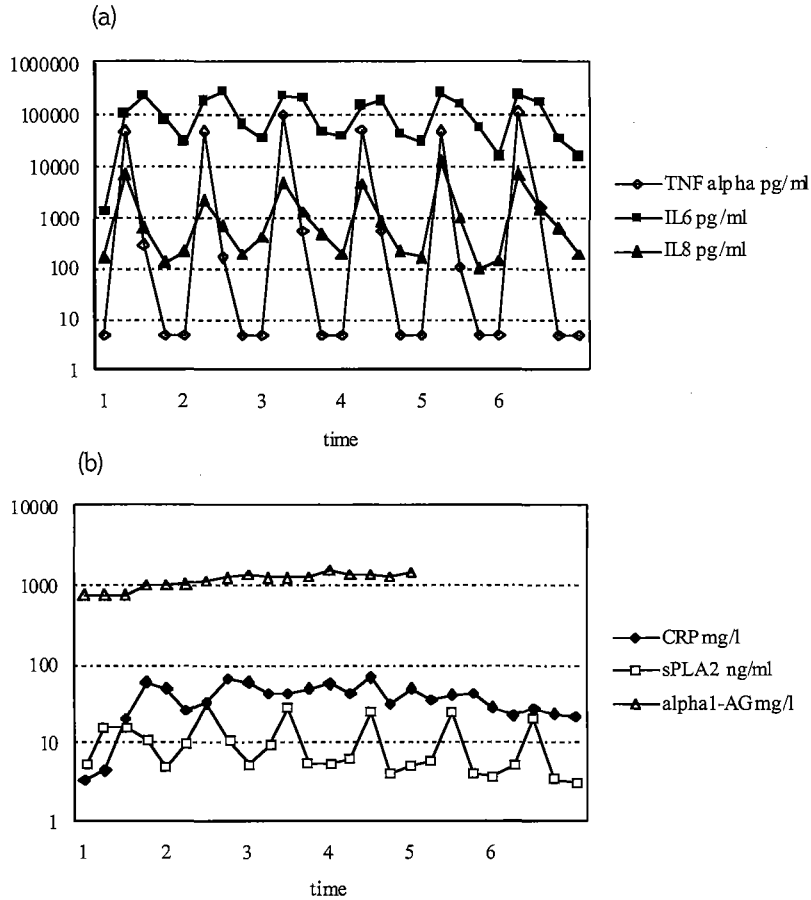


*Figure 1. Cytokine (a) and acute phase protein (b) profile in pleural fluid during the first 6 cycles of intrapleural TNF-administration in patient 2. Time-points: 24 hours before administration of TNFα, 24 hours after TNF-administration, 48 hours after, and 8 days after. Day 14 is 24 hours before the next gift of TNFα.*

*Toxicity and tumor response*

Five male patients were treated with intrapleural infusion of TNFα. Stage I patients were treated with 0.1 mg and one patient with stage IIA mesothelioma was treated with 0.2 mg. One patient received only one dose of TNFα: Because of excessive pleural effusion, for which the patient needed drainage of pleural fluid, complicated by a hematothorax, further intrapleural TNF-administration could not be pursued. The other 4 patients were evaluable for toxicity and tumor response as they received a minimum of 6 doses up to 18 doses. Overall tolerance was quite good. All patients developed flu-like symptoms and

mild fever (38-39.3°C) during 1-2 days. Two patients had a short episode of nausea and vomiting. No grade III-IV toxicity according to the WHO-criteria[28] was observed. No hypotension or increase in heart rate was observed. Furthermore, intrapleural TNFα had no effect on number of leukocytes and thrombocytes, kreatinin and liver functions.
None of the patients had a tumor response, only one patient showed stable disease during 36 weeks. Of note, once started with treatment none of the patients needed drainage of pleural fluid any more. In two patients dyspnea diminished obviously during treatment, in one patient dyspnea did not change and one patient did not have complaints of breathlessness.

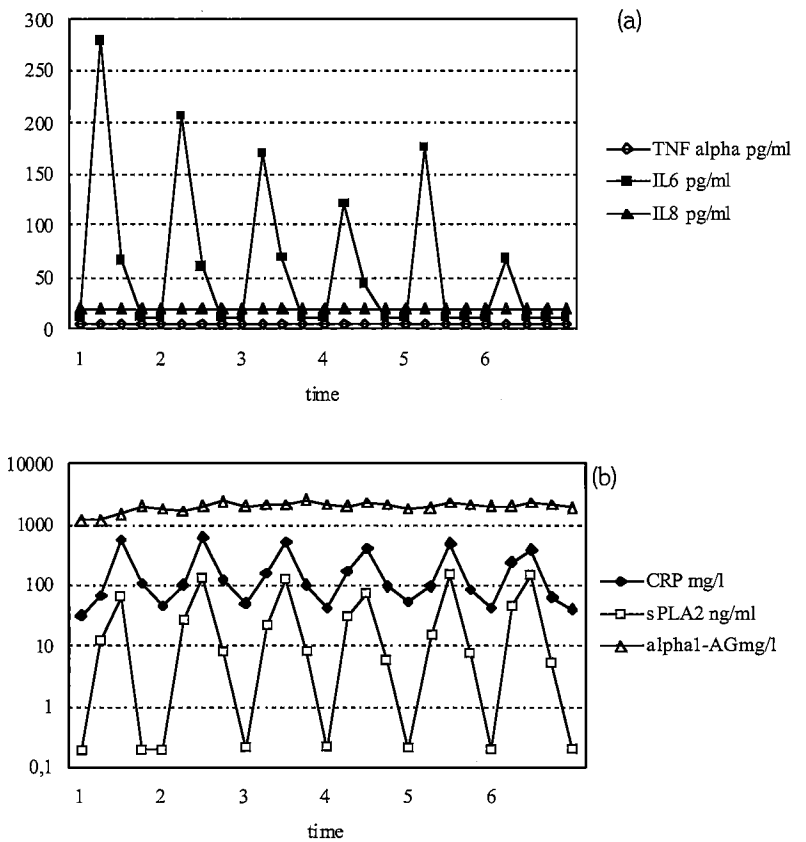


*Figure 2. Cytokine (a) and acute phase protein (a) profile in serum during the first 6 cycles of intrapleural administration of TNFα in patient 2. Time-points: 24 hours before administration of TNFα, 24 hours after TNF-administration, 48 hours after, and 8 days after. Day 14 is 24 hours before the next gift of TNFα.*

## DISCUSSION

In the present study the biologic effects of intrapleural administration of TNFα in patients with pleural mesothelioma was studied. The results of the cytokine measurements before administration of TNFα showed signs of an ongoing inflammatory response. This was also observed by others.[34,35] Before administration of TNFα, intrapleural levels of IL-6, IL-8, CRP, $sPLA_2$ and α1-AG were elevated. In serum we found increased levels of CRP and α1-AG. Our measurements correspond to the observations of Nakano et al. and Monti et al.[34,35] The high intrapleural IL-6 levels, before the first administration of TNFα, appeared to be caused by production by the mesothelioma-cells. IL-6 has been shown to be produced by mesothelioma-cells in vitro.[36,37] IL-6, produced in the pleural cavity, leaking to the systemic circulation, is thought to be the responsible cytokine for the induction of the APR. The mechanism of the APR has been investigated and described earlier, both in vitro[38,39] and in vivo.[26,40,41]

Intrapleural administration of TNFα resulted in clear IL-6, CRP, $sPLA_2$ and α1-AG patterns in serum, with the noticeable exception for TNFα and IL-8, which never reached detectable levels systemically. This may be explained by soluble receptors for TNFα and IL-8. With respect to the IL-6 response in serum a significant increase with a peak after 48 hours was observed after each TNF-administration. However, the response diminished gradually in the course of time, most illustrative visible in patient 2 in which no detectable IL-6 levels were observed when TNFα had been administered more than 9 times. This observation indicates an "exhaustion" phenomenon or a gradual build up of soluble IL-6 receptor levels.

However, the acute phase response was not diminished. This may be explained by a direct stimulation of the acute phase response by TNFα and IL-1.[26,40] TNFα and IL-1 can only stimulate the positive acute phase proteins. So in our study (we only determined positive acute phase proteins), we could not distinguish which cytokine had the most impact on the stimulation of the APR: IL-6 or TNFα/ IL-1. In contrast with the studies mentioned above[24-26], we could not find a downregulation of the APR, even though the acute phase response was already activated before the first TNF-administration. Administration of TNFα did increase the levels of the acute phase proteins; this indicates that the APR was not maximally stimulated before the first TNF-administration. A possible explanation for the fact we did not find a down-regulation of the APR could be the relatively long treatment free period of 2 weeks between the consecutive administrations of TNFα.

TNFα had no antitumor effect in these 4 mesothelioma-patients, but seemed to diminish pleural effusion. Reduction of pleural fluid was also described before.[21,49] The mechanism of this TNF-induced reduction of pleural fluid is not quite clear. Most likely, fluid production by mesothelioma cells is reduced. Enhanced resorption of pleural fluid is less likely as this mechanism would be associated with higher systemic levels of TNFα and other cytokines, which we did not observe.

In conclusion, intrapleural administration of TNFα was followed by a clear inflammatory response locoregionally. In spite of TNFα peak levels as high as 700 ng/ml, systemic levels were never detectable. The secondary cytokine response led to very high intrapleural IL-6 and IL-8 levels. IL-8 levels were never detectable in serum, whereas high IL-6 levels were induced systemically initially, with a decreased response to each intrapleural TNFα administration over time. The acute phase response in contrast remained remarkably constant throughout the course of repetitive intrapleural administrations of TNFα. Intrapleural administration of TNFα is well tolerated but associated with a rather moderate impact on production of pleural fluid. As this effect can be achieved by other simpler and cheaper treatment we see no justification for further studies.

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# CHAPTER 6

## REGULATION OF FERRITIN: A SPECIFIC ROLE FOR INTERFERON-ALPHA (IFNα)? THE ACUTE PHASE RESPONSE IN PATIENTS TREATED WITH IFNα-2B

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## ABSTRACT

**Background:** Adult onset of Still's disease is characterized by very high serum ferritin levels, in disproportion with other acute phase proteins (APPs). Since IFNα was observed to cause hyperferritinaemia in 3 healthy persons without increase of other APPs, we hypothesized that IFNα stimulates specifically the synthesis of ferritin. To test this hypothesis we studied ferritin- and other APP-levels in patients treated with IFNα.

**Patients and methods:** Fifteen patients treated with IFNα-2b 3-5 times a week, as adjuvant treatment after excision of a high-risk melanoma, were compared to 6 patients without adjuvant treatment (controls). Serum levels of C-reactive protein (CRP) and secretory phospholipase $A_2$ ($sPLA_2$) were measured using enzyme-linked immunosorbent assay (ELISA). Levels of ferritin, alpha1-acid glycoprotein (AAG) and albumin were determined by nephelometry.

**Results:** CRP was significantly decreased after 4 weeks ($p<0.01$) in the patients treated with IFNα compared with the non-treated patients, after 6 months of treatment it was still decreased although not significantly. Ferritin increased significantly in the IFNα-treated patients: 187% of pre-treatment value after 4 weeks and 217% after half a year ($p<0.01$), while ferritin levels decreased in the non-treated patients. AAG increased significantly in IFNα-treated patients (107, 114%) compared with the control-patients (91, 76%) but differences were less compared with CRP and ferritin. $sPLA_2$ had a variable course, while albumin remained constant within the normal range in both patient groups.

**Conclusions:** IFNα induced a significant increase in ferritin, with a significant decrease in CRP, little increase in AAG, varying response of $sPLA_2$ and no change in albumin. This finding suggests a specific role for IFNα in the synthesis or secretion of ferritin. This mechanism may also be involved in the marked hyperferritinaemia in adult onset of Still's disease.

## INTRODUCTION

The acute phase response (APR) is a non-specific reaction to injurious stimuli, including trauma, infection, malignant growth and inflammatory disorders. The main constituents of the APR, the acute phase proteins (APPs), are produced by hepatocytes.[1] The production of APPs is regulated by the local presence of cytokines. In the early stage of an inflammatory response these cytokines are released by activated monocytes and macrophages. From these cytokines interleukin-6 (IL-6), tumor necrosis factor-alpha (TNFα) and interleukin-1 (IL-1) are the major regulators of the production of APPs.[2-4] In clinical studies with patients treated with TNFα or IL-6 a strong relationship between the different APPs has been found.[5-8] A sharp increase of CRP and serum amyloid A (SAA) is observed, followed by increased levels of ferritin, protease-inhibitors and the complement factors, respectively.

This pattern is also seen in almost all rheumatic diseases, except for adult onset of Still's disease (AOSD). AOSD is the adult variant of the systemic form of juvenile arthritis. A marked feature of this disease is that in almost all patients very high serum ferritin levels are measured.[9-12] Normally a fixed correlation between CRP and ferritin is observed both in vitro and in vivo, with a CRP/ferritin ratio > 100 in favor of CRP.[13-15] With the high ferritin levels in AOSD there is dissociation between CRP and ferritin. The cause of the very high ferritin levels is still not known. Based on the findings of Baynes et al.[16] who found that administration of interferon-alpha in 3 healthy human subjects caused hyperferritinaemia, without concomitant rise of other APPs, we hypothesized a key-role for interferon-alpha in this phenomenon. To test this hypothesis we determined the APR in melanoma patients treated with IFNα. Special attention was given to ferritin, with the expectation that IFNα could preferentially stimulate the synthesis of ferritin.

## PATIENTS AND METHODS

*Patients and treatment*

The first 21 patients were selected out of a cohort that participates in a phase III study of the European Organization for Research and Treatment of Cancer (EORTC) of the adjuvant treatment with interferon-alpha2b (IFNα) in patients after operative treatment of a very high-risk melanoma (T4N0M0-TxN1-2M0). In that study treatment with subcutaneously (s.c.) administered high-intermediate dose IFNα is compared with treatment with low-intermediate dose IFNα and with patients without adjuvant treatment. Treatment with IFNα was started within 8 weeks after definitive surgical treatment. Treatment schedule and patient characteristics are summarized in Table 1.

*Table 1. Treatment schedule and patient characteristics.*

| | Group A | Group B | Group C |
|---|---|---|---|
| Treatment schedule | 5 days/week 10 MU IFNα for 4 weeks, followed by 10 MU IFNα 3 days/ week for 1 year | 5 days/week 10 MU IFNα for 4 weeks, followed by 5 MU IFNα 3 days/ week for 2 years | Observation |
| Number of patients | 7 | 8 | 6 |
| Mean age (range) | 44 (20-61) years | 42 (27-66) years 55 | (26-72) years |
| Sex male/ female | 2/5 | 2/6 | 5/1 |

*Blood sampling procedure*

Venous blood samples of these patients were collected before start of the treatment with IFNα, 4 weeks after start of treatment (at the end of the "induction period" consisting of 10 MU, s.c., 5 days/week in both group A and B), and 6 months later (during "maintenance treatment" consisting of 10 MU of IFNα (group A) or 5 MU of IFNα (group B), s.c. 3 times/week). The patients in the control arm of the study (no IFNα) were sampled at the same time-points. Serum samples were kept frozen at -20°C until assay.

*Assays for acute phase protein analysis*

Levels of CRP and $sPLA_2$ were determined using enzyme-linked immunosorbent assay (ELISA). Used antibodies (Abs) were obtained from the Central Laboratory of the Blood Transfusion Service (CLB, Amsterdam, The Netherlands). CRP levels were measured by

a sandwich ELISA using polyclonal rabbit anti-human CRP Abs as catching Abs and biotinylated monoclonal antibody (mAb) anti-CRP (CLB anti-CRP-2) as a detecting Ab.[17] Results were referred to a standard (Behringwerke AG, Marburg, Germany) and expressed in mg/l. Detection limit was 10 ng/l. Normal values (obtained from 100 blood donors) were < 5 mg/l.

The ELISA used for measuring $sPLA_2$ was described before.[18] Two different mAbs against human $sPLA_2$ were used as coating and catching Abs respectively. The lower limit of detection was 0.1 ng/ml. Normal healthy volunteers were < 5 ng/ml.

Levels of ferritin, AAG and albumin were measured by means of a nephelometric assay. The used antisera were also obtained from the CLB. Normal values were ferritin 20-150 µg/l; AAG 0.4-1.3 g/l; albumin 35-55 g/l.

**Statistics**

The Mann-Whitney U test was used to compare the 2 groups depicted in Table 2. For comparison of the ratios of CRP and ferritin (Table 3) we used the Fisher's exact test. A p-value of $< 0.05$ was regarded as significant.

## RESULTS

Median values with range of all measured acute phase proteins, expressed as percentages of pre-treatment levels, both in IFNα-treated patients and in control-patients, are depicted in Table 2. Since the results of the measurements in group A (maintenance treatment at 10 MU IFNα) and B (5 MU IFNα) were not significantly different, the groups were taken together. Group A+B was compared with the control-group by calculating significance between the values after 4 weeks (end of 10 MU IFNα 5x/wk in both groups) and 6 months (10 MU IFNα, 3x/wk in group A, 5 MU IFNα 3x/wk in group B) in group A+B and group C.

*Table 2. Median (range) of change acute phase proteins expressed as a percentage of the pre-treatment value in high-risk melanoma-patients getting adjuvant treatment with subcutaneous IFNα, compared with patients without adjuvant treatment. Significance was calculated using the Mann-Whitney U test, comparing data of both groups after 4 weeks and 6 months.*

| | **Group A+B** | Range | **Group C** | Range | p-value |
|---|---|---|---|---|---|
| **CRP** | | | | | |
| 4 wk | 25 | 2-133 | 178 | 91-533 | p<0.01 |
| 1/2 yr | 46 | 3-90 | 82 | 21-500 | n.s. |
| **Ferritin** | | | | | |
| 4 wk | 187 | 83-1269 | 86 | 56-233 | n.s. |
| 1/2 yr | 217 | 69-417 | 68 | 40-144 | p<0.01 |
| **sPLA2** | | | | | |
| 4 wk | 92 | 23-198 | 71 | 8-111 | n.s. |
| 1/2 yr | 88 | 26-213 | 59 | 13-132 | p<0.05 |
| **AAG** | | | | | |
| 4 wk | 107 | 83-190 | 91 | 77-105 | n.s. |
| 1/2 yr | 114 | 70-126 | 76 | 54-87 | p<0.01 |
| **Albumin** | | | | | |
| 4 wk | 93 | 93-100 | 95 | 92-102 | n.s. |
| 1/2 yr | 105 | 95-107 | 102 | 100-107 | n.s. |

*CRP, C-reactive protein; $sPLA_2$, secretory phospholipase $A_2$; AAG, α1-acid glycoprotein. Time-points: 4 weeks, 4 weeks after start of treatment with IFNα (at the end of the "induction period"); 1/2 year, 6 months later (during "maintenance treatment").*

CRP was significantly decreased in the patients treated with IFNα after 4 weeks. Values were also lower after 6 months of treatment compared to control-patients, although the difference was not significant. At the same time points CRP was increased in the control-group. Ferritin showed a reverse pattern: strong increase in IFN-treated patients, decrease in controls. AAG-levels resembled the pattern of ferritin-levels but with a less steep increase, respectively decrease. The higher pre-treatment values of ferritin and AAG in group C were not significantly increased compared with group A+B. Levels of $sPLA_2$ showed a variable course in the patients of group A+B and a decrease in the control-patients and albumin-levels remained constant in both patient-groups.
To avoid a wide range of all the determinations we additionally calculated the ratios of increase/decrease (compared to the pre-treatment value) for CRP and ferritin. With these ratio the relative increase/decrease was determined. In Table 3A the median (with range) values of the ratio are depicted and in Table 3B the results of the 2 groups were compared and significance was calculated. This method of representation confirmed the finding of a significant decrease of CRP with a concomitant increase of ferritin in the patients treated with IFNα compared to patients without adjuvant treatment.

*Table 3A. Median (with range) of CRP- and ferritin-ratios and relative increase.*

| ***4 wk*** | $CRP_{4wk}/CRP_{pre}$ | $Ferritin_{4wk}/ferritin_{pre}$ | Ferritin ratio/CRP ratio |
|---|---|---|---|
| A+B | 0.25 (0.03-1.3) | 1.7 (1.0-3.6) | 8.3 (1.1-91) |
| C | 1.8 (0.91-5.0) | 0.62 (0.48-1.0) | 0.32 (0.13-1.13) |
| ***½ yr*** | $CRP_{1/2yr}/CRP_{pre}$ | $Ferritin_{1/2yr}/ferritin_{pre}$ | Ferritin ratio/CRP ratio |
| A+B | 0.46 (0.03-9.0) | 2.8 (0.69-4.2) | 4.6 (0.24-32) |
| C | 0.91 (0.05-5.0) | 0.68 (0.40-2.3) | 1.3 (0.19-13) |

*Table 3B. Comparison of CRP- and ferritin-ratios and relative increase between patients treated with IFNa and control-patients.*

| ***4 wk*** | $CRP_{4wk}/CRP_{pre}$ # | | $Ferritin_{4wk}/ferritin_{pre}$# | | Ferritin/CRP ratio * | |
|---|---|---|---|---|---|---|
| | ≥1 | <1 | ≥1 | <1 | ≥1 | <1 |
| A+B | 2 | 13 | 15 | 0 | 15 | 0 |
| C | 3 | 1 | 2 | 2 | 1 | 3 |
| ***½ yr*** | $CRP_{1/2yr}/CRP_{pre}$ ‡ | | $Ferritin_{1/2yr}/ferritin_{pre}$# | | Ferritin/CRP ratio # | |
| | ≥1 | <1 | ≥1 | <1 | ≥2 | <2 |
| A+B | 5 | 10 | 12 | 3 | 11 | 4 |
| C | 3 | 3 | 1 | 5 | 1 | 5 |

*CRP, C-reactive protein; Time-points: pre, before start of treatment with IFNα; 4 weeks, 4 weeks after start of treatment (at the end of the "induction period"); $^1/_2$ year, 6 months later (during "maintenance treatment"). #: p<0.05; *: p<0.01; ‡: not significant*

## DISCUSSION

In the present study we observed a significant increase of ferritin after 4 weeks and 6 months in patients treated with IFNα, with a less pronounced effect on CRP, AAG and $sPLA_2$ and no effect on albumin. The increase of ferritin in our patients was comparable with the observations of Baynes et al.16 who described an increase of 170% (percentage of basal value) in 3 healthy human subjects after a single subcutaneous injection of IFNα. CRP was also measured: it remained within the normal range.

Besides this study, describing the short-term effect of IFNα-therapy we found one case-report describing long-term administration of IFNα.[19] In a patient with chronic hepatitis C and liver hemosiderosis treatment with IFNα during 24 weeks resulted in a transient increase of ferritin during the first 3 weeks followed by a decrease. These observations were in discrepancy with our findings. However, the situation in the patient of the case report is more complicated: before treatment his ferritin-level was 1250 μg/l, while in our patients levels of ferritin were not increased before treatment.

In hepatitis patients the increased ferritin levels could be a reflection of the eventual ongoing acute phase reaction. Moreover in several studies the increase of ferritin levels in hepatitis patients correlated with an iron overload (tissue levels).[20,21] In that sense by treating the disease the balance between inflammatory reaction in the liver/ iron content next to the possible effect of IFNα on the postulated increase of ferritin synthesis and production will determine the ferritin levels in these patients. By treating hepatitis patients with iron chelating therapy and IFNα the serum ferritin levels decrease far more significant, but still the ferritin levels were reported as slight increased above the upper limit of normals.[20]

CRP and ferritin both behave as positive acute phase proteins, as was illustrated in our previous studies with cancer patients who underwent isolated limb perfusion with TNFα.[6] In our patients treated with IFNα we observed an inverse relation between both acute phase proteins. Our observations suggest that IFNα might preferentially stimulate synthesis and/or production of ferritin. But we realize that until now no ex-vivo experiments are performed which will sustain our conclusions.

A possible factor of influence on levels of the acute phase proteins is disease activity,

maybe changed by treatment with IFNα. Comparison of erythrocyte sedimentation rate (ESR) and the number of leukocytes in treated patients and in control patients (data not shown) showed no significant changes. Furthermore the sex ratio in the treated group was different from the sex ratio in the control group. We included the first patients treated in this trial, so this difference was inevitable. Since we found no significant difference in the pre-treatment ferritin-levels between the 2 groups we accepted the difference in sex ratio.

The mechanism of the increase in ferritin after administration of IFNα is still not clear. As the other APPs did not (or little) increase, a role for the acute phase response is not likely. Since an increase of hepatic enzymes was reported after IFNα therapy[22-24], release of ferritin caused by liver damage could be an explanation for the increased serum ferritin levels. This mechanism was also suggested in AOSD.[25,26] In our patients 5 out of 15 IFN-treated patients had grade I-III increase of transaminases but without concomitant rise of LDH (data not shown). So release of ferritin caused by liver damage is not a probable explanation. Thus release of ferritin after administration of IFNα seemed to be provoked by a direct effect of IFNα.

In conclusion, the results of this study showed a significant increase of ferritin without comparable increase of other positive APPs after administration of recombinant IFNα. These findings supposed a specific role for IFNα in the synthesis or secretion of ferritin. This phenomenon could also be involved in the extremely high levels of ferritin in adult onset of Still's disease.

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# CHAPTER 7

## GENERAL DISCUSSION

## 7.1 Introduction

Tumor necrosis factor-alpha (TNFα) is a proinflammatory cytokine with a broad spectrum of biological activities. Its name is derived from the ability to induce tumor cell necrosis. Since recombinant TNFα became available in large quantities[1], many efforts were undertaken to make use of its antitumor effect in man. In murine tumor models TNFα induced tumor necrosis with acute softening of the tumor, hemorrhagic necrosis and occlusion of the tumor vasculature.[2,3] These promising results led to the application of TNFα in phase I/II trials in the treatment of cancer. Unfortunately, systemic administration of TNFα was limited by severe toxicity at very low concentrations of TNFα without evident antitumor effect. Hypotension was the dose-limiting factor. Given these findings many attempts were undertaken to apply TNFα as a local treatment. Intratumoral[4,5], intra-arterial[6,7] and intracavitary[8,9] administration had little to no antitumor effect, again toxicity was dose limiting. Only 1/50 to 1/20 of the dose required for antitumor effect in human xenograft tumor models in mice could be administered in man.
The problem of the dose-limiting toxicity could be overcome by the isolated limb perfusion (ILP) technique. The first results of ILP with TNFα in combination with melphalan and interferon-gamma (IFNγ) were published in 1992 by Liénard and Lejeune.[10] This treatment resulted in a 90% complete response rate in patients with in transit metastasized melanoma[10-12] in contrast to a 50% CR rate after ILP with melphalan alone.[13,14]
In locally advanced extremity soft tissue sarcomas limb salvage was achieved in > 80%.[15,16] The efficacy of TNFα against the drug-resistant soft tissue sarcomas has led to the approval of TNFα by the European Medicine Evaluation Agency (EMEA) for its use in combination with melphalan.[17]
The application of TNFα in the ILP-setting enabled us to investigate the effects of short-term exposure to TNFα and melphalan in man.

## 7.2 Effects of TNFα on white blood cell-populations and cell-function

### *7.2.1 Effects of TNFα on white cell-populations*

The procedure of ILP performed in patients with locally advanced melanoma or sarcoma results in remarkably little toxicity in spite of transient considerable systemic levels of TNFα in the first couple of hours after ILP. To get more insight in the effects of leakage of TNFα to the systemic circulation during ILP, we investigated changes in cell

population and cell function in patients who underwent ILP with TNFα and melphalan. We determined the number of monocytes and lymphocytes in patients undergoing ILP with TNFα and melphalan at several time-points during and after ILP, and compared the results with the same measurements in patients who underwent minor or major surgery. ILP with TNFα and melphalan resulted in a significant decrease in the number of monocytes and a minor decrease of lymphocytes during ILP till 3 hours after ILP, followed by a fast restoration within one day.[18] In patients who underwent major or minor surgery we did not observe significant changes in numbers of monocytes of lymphocytes. So a confounding effect of surgery or anaesthesia on changes in cell populations could be excluded.

Several reports in the literature have described the effects of intravenous administration of TNFα on leukocytes and subpopulations. TNFα has been shown to induce a precipitous leukopenia within 30 minutes after administration.[19-21] Differentiation of the subpopulations of leukocytes gave the following observations: Neutrophils declined within a few minutes, followed by a sharp increase in neutrophil counts. Monocytes also decreased rapidly, reaching lowest levels after 30 minutes. Lymphocytes and eosinophils decreased during the first 4 hours after administration of TNFα.[19,20,22,23]

The initial neutropenia is most likely caused by adhesion of neutrophils to the vascular endothelium. In in-vitro studies TNFα was found to enhance the adherence of neutrophils to cultured endothelial cells. Neutrophils adhere to vascular endothelium by increasing the expression of the CD11/18 complex on the surface of neutrophils[24-26] and by endothelial cell adhesion molecule expression.[25,27] ICAM-1 (intercellular adhesion molecule-1), E-selectin and P-selectin (as endothelial cell adhesion molecules) were found to be involved in the TNF-induced adhesion of neutrophils to the endothelial cells. The expression of selectins increased with increasing concentration of TNFα.[25]

The subsequent neutrophilia consisted of increased numbers of immature neutrophils, suggestive of recruitment of neutrophils from the bone marrow.[28,29] Adrenalectomized rats that were treated with TNFα developed the same pattern of neutropenia and neutrophilia, indicating that TNFα did not mediate its hematologic effects via the release of adrenal hormones.[29]

Molecular mechanisms of monocyte adhesion are less well understood. Several endothelial cell adhesion molecules (VCAM-1, vascular cell adhesion molecule-1; ICAM-1) and monocyte integrins (LFA-1, lymphocyte function antigen-1 (CD11a/CD18); Mac-1, CD11b/CD18; VLA-4, very late antigen-1) had been shown to be involved.[30-32] The TNFα-induced lymphopenia and eosinophil adhesion is also based on an interaction of

surface-proteins on lymphocytes and endothelial cells.[33-36] A clear difference was found between T-cell and neutrophil adhesion to TNF-stimulated endothelial cells in in vitro adhesion assays: Adhesiveness of neutrophils increased rapidly within 4 hours, while adhesiveness for T-cells was smaller and more gradual.[37]
The rapid onset of induction of adhesion molecules on endothelial cells and integrins on leukocytes pointed out a direct effect of TNFα. Nevertheless influence of other mediators, for instance other cytokines, induced by TNFα can not be excluded.
The activation of endothelial cells by TNFα is not specific for tumor microvasculature. Activated endothelial cells were also found in normal vessels after exposition to TNFα.[38,39] Activation of tumor endothelial cells leads to overexpression of adhesion molecules, followed by invasion of polymorphonuclear cells and possibly lymphocytes and macrophages, endothelium injury, and coagulative and haemorrhagic necrosis.[40,41] In normal tissue the upregulation of adhesion molecules was not followed by polymorphonuclear cell invasion and necrosis. The underlying mechanism of this difference between tumor endothelial cells and normal tissue endothelial cells is still under investigation. A possible role is ascribed to integrin αVβ3, which is expressed by endothelial cells during formation of new blood cells, but nor by resting endothelial cells. Rüegg et al. demonstrated that TNFα and IFNγ could inhibit αVβ3. This resulted in disruption of endothelium in the tumor vasculature.[42]
Both in ILP-patients and in patients treated with TNFα intravenously TNFα induced a fast disappearance from the systemic circulation of all types of leukocytes, by inducing adhesion to the endothelial cells. This mechanism plays an important part in the migration of cells into sites of inflammation.

### *7.2.2 Effects of TNFα on cell function*

Cell function in our patients was determined by measuring production of cytokines after stimulation in whole blood cell culture (WBCC). For monocyte-function TNFα and IL-6 were determined after stimulation with lipopolysaccharide. In patients treated with ILP we found a significant decrease in cytokine-production which started already before the administration of TNFα (possibly caused by haemodilution). Cytokine production remained significantly diminished until day 1. In patients who underwent major surgery we observed also a decrease in cytokine production which started during operation. However, the reached nadirs were higher compared to the levels in ILP-patients, and the cytokine production was restored within one day. After minor surgery and in healthy controls no fluctuations in cytokine production were observed.[43]

Lymphocyte-function was determined by measurement of IL-8 production after whole-blood stimulation with anti-CD3/anti-CD28. In an additional study we tried to distinguish between T-helper-1 (Th1) and T-helper-2 (Th2) cells, by measuring intracellular levels of IFNγ (produced by Th1-cells) and IL-4 (produced by Th2-cells) after stimulation in a culture of peripheral blood mononuclear cells. We also measured IFNγ, IL-2 (Th1) and IL-4 (Th2) after stimulation in WBCC.

In ILP-patients and in patients who underwent major surgery we observed a decrease in cytokine-production from the end of the operation until day 1. After minor surgery and in healthy controls we observed no change in cytokine-production.[18] Intracellular staining of cytokine production showed a decrease in IFNγ-producing cells, from the time-point of administration of TNFα into the perfusate until 3 hours after ILP. The fraction of IL-4-staining cells was too small to measure fluctuations during and after ILP.[43]

Our in-vivo findings are quite different from observations made in in vitro systems: cytokine-production in monocyte cultures in response to lipopolysaccharide (LPS) could be augmented by TNFα.[44,45] Similarly, as was found for monocytes, TNFα also enhanced in vitro production of cytokines by T-lymphocytes.[46-48]

A decreased monocyte and lymphocyte function was found in patients after intravenous administration of TNFα.[49,50] Furthermore, in patients after major surgery[51-53] and during sepsis[54,55] downregulation of cytokine production by monocytes and lymphocytes had been described.

For explanation of the monocyte-deactivation several hypotheses have been suggested: Tissue injury by itself is certainly the key factor in immunodepression and altered cytokine production after surgery. This is supported by the observations that patients with extensive burns[56] and patients with multiple accidental injuries[57,58] also have severe immunosuppression, manifested in depressed in vitro cytokine production by monocytes. Tissue injury leads to activation of macrophages and production of proinflammatory mediators. This local inflammatory reaction is supposed to induce several cascades resulting in monocyte deactivation.

First, local or systemic inflammation leads to activation of the stress response, expressed by corticosteroids and catecholamines. These stress hormones induce an upregulation of IL-10.[59] IL-10 was demonstrated in vitro to be a potent macrophage-deactivating cytokine.[60-62] Also in vivo IL-10 was found to play a key-role in the several mechanisms of immunosuppression in patients with septic shock[63] and in patients undergoing major abdominal surgery.[64]

During inflammation TNFα by itself can induce IL-10 mRNA as well, and in combination

with other stimuli (like low-dose LPS or prostaglandins) it can also induce IL-10 protein synthesis, leading to monocyte deactivation.[59] In this way inflammation may trigger its own downregulation.

A third hypothesis is based on the finding that uptake of apoptotic material by macrophages inhibits the release of inflammatory cytokines by human macrophages.[65-67] Massive induction of apoptosis (e.g. in organ failure in septic and trauma patients) can thus result in a downregulation of TNF secretion capacity and upregulation of IL-10 secretion.[59]

*Table 1. Mechanisms of monocyte-deactivation in patients treated with TNFα*

1. Stress-response (corticosteroids and catecholamines) induces IL-10
2. TNFα induces IL-10
3. Massive uptake of apoptotic material by monocytes upregulates IL-10 secretion

The biological function of the endotoxin hyporesponsiveness can be assumed to be protection against excessive inflammatory response. This results in an increased risk to develop sepsis and multiple organ failure in patients with affected monocyte function.[51,68] As was found in our ILP-patients a downregulation of T-cell cytokine-production was also demonstrated in patients undergoing various types of trauma such as surgery or burn injury and in patients with septic shock. Major surgery[69-71], trauma[72] and severe sepsis[73] decreased the number of T-helper cells and induced a shift in the T-helper 1/ T-helper 2 (Th1/Th2) balance toward Th2, caused by decrease of Th1-cells. Besides decrease of the number of T-helper cells also the cytokine-production of the remaining cells after in vitro stimulation was diminished.[70,71,74] This suppression of cellular immunity was found to be caused by hypersecretion of catecholamines, cortisol and other adrenal corticosteroids. Also overproduction of immunosuppressive acidic protein and other acute phase reactants[69,75,76] and production of $PGE_2$ from activated monocytes/ macrophages[77,78] has been reported to result in diminished T-cell cytokineproduction.

Since TNFα is a known inducer of cortisol[23,79], $PGE_2$[80-82] and the acute phase response, the mechanisms mentioned above can also be involved in the immunosuppression following TNF-administration.

*Table 2. Mechanisms of impairment of lymphocyte function in patients treated with TNFα*

1. Stress-response (corticosteroids and catecholamines)
2. Production of immunosuppressive acidic protein and other APPs
3. $PGE_2$, produced by activated monocytes

Exposure to TNFα in ILP-patients was shown to induce a transient impairment in monocyte and lymphocyte function. Comparable observations have been made in patients who underwent major surgery or patients during sepsis. Several causal factors had been hypothesized in the mechanism of this immunosuppression. Since cancer patients and non-cancer patients peri- and postoperative are especially in need of their immune system against respectively growth and metastasis of residual tumor cells and post-operative infections immunosuppression must be prevented as much as possible. In the future it may be possible to intervene in the mechanism of immunosuppression with biological response modifiers to reduce the incidence of postoperative-complications.

## 7.3 TNFα and the acute phase response

*7.3.1 The acute phase response after ILP*

TNFα is one of the inflammation-associated cytokines that are involved in the regulation of acute phase protein (APP) synthesis. Interleukin-6 (IL-6) and IL-6 type cytokines are the chief stimulators of the acute phase response by stimulating the full spectrum of acute phase proteins, both upregulation of positive APPs and downregulation of negative APPs. TNFα as well as interleukin 1 (IL-1) (together IL-1 type cytokines) have only effect on a subset of positive APPs[83-85] and negative APPs.[86] This classification is based on in vitro studies.

In our patient groups we could determine the specific role of TNFα as a stimulator of the acute phase response (APR) in vivo. Our observations in the systemic circulation of patients undergoing an ILP had the following results: high systemic levels of TNFα directly after ILP induced a rapid increase of IL-6 and IL-8. C-reactive protein (CRP), secretory phospholipase $A_2$ ($sPLA_2$), α1-acid glycoprotein (AAG) and α1-anti-trypsin (AAT), complement components C3 and C4 started to increase 3 hours after the end of ILP. The highest levels of CRP and $sPLA_2$ were obtained 1-2 days after ILP. The serum levels of the other APPs increased more slowly and modestly and reached their highest

level at day 3. All investigated proteins declined after they had reached their peak levels, except for C3 and C4 which both remained elevated for at least 4 days. The negative APPs, albumin and transferrin decreased to a minimum of 60% of their initial value at 10 minutes after ILP. The recovery was very slow; albumin did not reach its initial value within 7 days.[87-89] Most observations were done in patients who underwent an ILP without systemic leakage during the perfusion. We also performed some determinations in patients who underwent an ILP with high systemic leakage (12-65% leakage). High systemic levels of TNFα resulted in ten- to more than hundredfold increased levels of TNFα, IL-6 and IL-8. The increase of the levels of the APPs CRP and $sPLA_2$ was less pronounced; CRP-levels were even lower compared to the levels in patients without leakage.

As already mentioned above, the patients with high systemic leakage had a marked mild clinical course. The temporary hypotension could be easily dealt with by fluid challenge and sometimes by temporary vasopressor support. This relative lack of toxicity can be explained by the short duration of exposure to elevated levels of TNFα in comparison with septic shock. Moreover, septic patients are infected and have significant levels of endotoxin, which has been shown to be synergistic with TNFα for toxicity. Adequate diuresis is very important to keep the high circulating TNF levels as short as possible. This is a fundamental difference with the often poorly hydrated patients with metastatic cancer who received intravenous TNFα in phase I-II studies in the past. Finally, a protection mechanism was found in the soluble TNF receptors, which are shown to buffer the bioactivity of TNFα.[90]

Most likely the observed alterations in acute phase protein levels reflect the fluctuations in the synthesis of the APPs by the liver, induced by the activated cytokine network. In vitro, TNFα stimulates the synthesis of various positive APPs (AAG, serum amyloid A (SAA), CRP, C3) and decreases synthesis of the negative APPs albumin and transferrin in human hepatoma and rat hepatoma cell culture.[91-93] In rodents, administration of TNFα resulted in increase of SAA, serum amyloid P (SAP), C3, AAG and fibrinogen.[92,94-97] The effect of TNFα was found to be mediated by the type I TNF receptor (p55).[91,98]

Although these findings correspond largely to our results in humans we have to realize that the in vivo situation is much more complex, since in vivo hepatocytes are exposed to a sequence and combination of interacting cytokines and other mediators.[99]

In the in vitro and in vivo findings mentioned above besides type 1 APPs (induced by IL-1 type cytokines) some type 2 APPs (induced by IL-6 type cytokines) are mentioned. Also

in our ILP-patients we found an increase in AAT, which is a type 2 APP. This indicates that we can not clearly distinguish the separate effect of TNFα on the measured acute phase protein levels, because of the rapid induction of other cytokines.
From our observations in the patients with high leakage of TNFα in the systemic circulation it can be suggested that acute phase proteins can not be stimulated above a certain level. This was also found in patients who underwent an isolated hepatic perfusion for irresectable colorectal liver metastases.[100] To our knowledge this phenomenon has not been described in other clinical studies. However a reduction in CRP-levels had been well observed in malnourished patients who underwent a major abdominal operation, without a concomitant reduction in other APPs (AAG, AAT).[101,102] Also in rats receiving a protein deficient diet the magnitude and time profile of levels alpha2-macroglobin and albumin was attenuated.[103,104] Taking together these findings it can be concluded that particularly for CRP there is evidence that production seemed to be limited, probably caused by a shortage of protein-elements in the liver.
Our observations in the ILP-patients have yielded some more insight in the role of TNFα in the stimulation of the APR. ILP with TNFα and melphalan in patients with melanoma or sarcoma resulted in a rapid increase of many positive APPs and decrease of negative APPs. From data of patients with high systemic leakage it can be concluded that levels of APPs have a limited range.

*7.3.2 The acute phase response during repeated/chronic stimulation*

The preceding studies describe the acute phase response after a relative short-term exposure to TNFα. Since until now little is known about the conversion of the acute phase response to chronic inflammation we wanted to study a clinical model with long-term administration of TNFα in patients. A phase I trial in which patients with malignant mesothelioma were treated with repeated intrapleural administration of TNFα presented the opportunity to study this question.
Every 2 weeks administration of TNFα in the pleural cavity resulted in a locoregional cytokine- (TNF, IL-6, IL-8) and APP- (CRP, $sPLA_2$, AAG) response. In pleural fluid TNFα was measurable for more than 48 hours. IL-6, IL-8, CRP and AAG remained elevated till the next administration of TNFα. In serum no detectable levels of TNFα and IL-8 could be measured. IL-6 was elevated during more than 48 hours, although the magnitude of the values decreased after every TNF-injection. The decreasing response of IL-6 was accompanied by a clear and stable acute phase response.[105]
Normally the APR subsides over 24-48 hours and within a few more days the organism returns to normal function. However, when the stimulus persists, the normal pathway is prolonged and the APR is supposed to be converted in a chronic phase of inflammation.

Several studies have been performed and reported on in the literature to get more insight in the mechanism of the continuation of the APR in chronic inflammation: Long-term culture of human hepatocytes or hepatoma cells resulted in a clear APR after exposure to IL-6. Peak-levels were reached within 2-4 days, followed by stabilization. After removal of the stimulus levels of APPs returned to pretreatment values within 5 days.[106-108]
Discrimination between acute and chronic inflammation in rodents and cattle was possible by measurement of different levels of elevated APPs: in cattle a differentiation could made in level of SAA and haptoglobin.[109] In mice concentration of AAG- and albumin mRNA were specifically different in acute and chronic inflammation.[110] After prolonged inflammation induced by intraperitoneal injections with turpentine, lipopolysaccharide and celite an initial increase of APP mRNA was followed after about 2 days by a gradual decline to a new baseline after 5 days.[110] A diminished APR after recurrent sterile inflammation in mice could not be demonstrated by Todd et al.[111] They suggested that the phenomenon of low APR in several chronic or recurrent inflammatory diseases in man may not simply result from chronic inflammation itself but may be part of a predisposition to chronic inflammation.
Clinical studies describing the APR in recurrent or chronic inflammatory diseases are far from unanimous. Some studies described a diminished APR (a discrepancy between cytokine-levels and levels of APPs) in multiple myeloma-patients[112], cachectic patients with pancreatic cancer[113] and in cancer-patients treated with subcutaneous IL-6.[114] Other studies did not observe a clear decrease in the levels of APPs in patients with advanced pancreatic cancer[115], in cancer-patients treated with subcutaneous recombinant human IL-6[116] and in patients with unstable angina pectoris.[117]
The fact that we did not find a downregulation of the acute phase response is maybe due to the relatively long time (14 days) between the consecutive administrations of TNFα. Since cytokines have a short half life and are only in circulation at important levels for a short time, the time between two successive cytokine-administrations is required to be within a few days. Nevertheless it is astonishing that comparable clinical studies resulted in such different observations as described above. This indicates that the APR is a complex system with multiple interactions between cytokines and APPs.
From our observations in patients who were treated with intrapleural administration of TNFα it can be concluded that repeated exposure to TNFα evoked a clear locoregional and systemic cytokine- and acute phase response. We did not observe a down-regulation of the APR in these patients in spite of the repeated inflammatory stimuli.

*7.3.3 IFNα and the acute phase response*

IFNα is known for its antiviral activity and its role in the treatment of malignancies.[118-120] Furthermore several clinical studies described induction of cytokines by IFNα.[121,122] Only a few studies had investigated the relation of IFNα to the acute phase response. Administration of IFNα in cancer patients had no effect on serum cytokines and APPs (CRP, AAG) in a study of Israel et al.[123] Bíró et al. described the response of the acute phase complement component to IFNα in patients with chronic hepatitis C.[124] In these patients the complement system was already activated before treatment and when administration of IFNα was started the intensity of activation decreased in both responders and non-responders. However only in the responder patients did this decrease result in an increase of serum concentration of complement proteins C9 and C1-inhibitor. So in these patients it seems IFNα has an indirect effect on the APPs, by influencing disease-activity.

Ferritin is a known acute phase protein, which is also involved in the iron-metabolism.[125-127] Patients with adult onset of Still's disease (AOSD) are characterized by very high-elevated ferritin-levels. Also levels of other APPs are increased, but ferritin levels are much higher in AOSD than in inflammatory diseases.[128-130] The origin of the great amount of serum ferritin in AOSD is still unknown. Levels of the cytokines TNFα and IL-6 are found to be increased in patients with active AOSD.[131,132] However this does not explain why ferritin is much more elevated than other APPs. An indication was found in observations in persons treated with subcutaneous administration of IFNα. In these persons a significant increase of ferritin was observed without a concomitant elevation of CRP.[133] Based on these findings we hypothesized a specific role for IFNα in the induction of hyperferritinaemia. To test this hypothesis we determined several levels of APPs in patients with high-risk melanoma during treatment with subcutaneously administered IFNα.[134] In these patients we observed indeed a significant increase in ferritin, accompanied with a significant decrease in CRP and little or no change in the other APPs. There was no difference between the effect of a dose of 5 MU IFNα and 10 MU IFNα.

The mechanism of the specific induction of ferritin by IFNα is still unknown. From our findings it was concluded that most possibly there must be a direct effect of IFNα on the synthesis or secretion of ferritin. Release of ferritin as participant in the APR could be excluded since other APP-levels showed little or no alteration. Also liver damage as a cause of hyperferritinaemia is less probable since only 5 out of 15 patients had grade I-III increase of transaminases, without concomitant elevation of LDH.

IFNα elicits its antiviral and immunoregulatory activities by binding to specific receptors on the cell surface.[118,135,136] These receptors are present on several cells, like monocytes, lymphocytes and hepatocytes.[136,137] The binding of IFNα to the cell surface receptor initiates signals that are transmitted from the cell surface via a complex pathway to the nucleus[118,138], resulting in multiple biological activities mediated by IFNα. A possible explanation of the increased ferritin in our patients could be the stimulation of secretion or synthesis of ferritin mediated by this system, since ferritin is also known to be present in the liver and in monocytes.[126,139]
Most possibly the increase of ferritin in patients treated with IFNα without a clear APR is direct effect of IFNα.

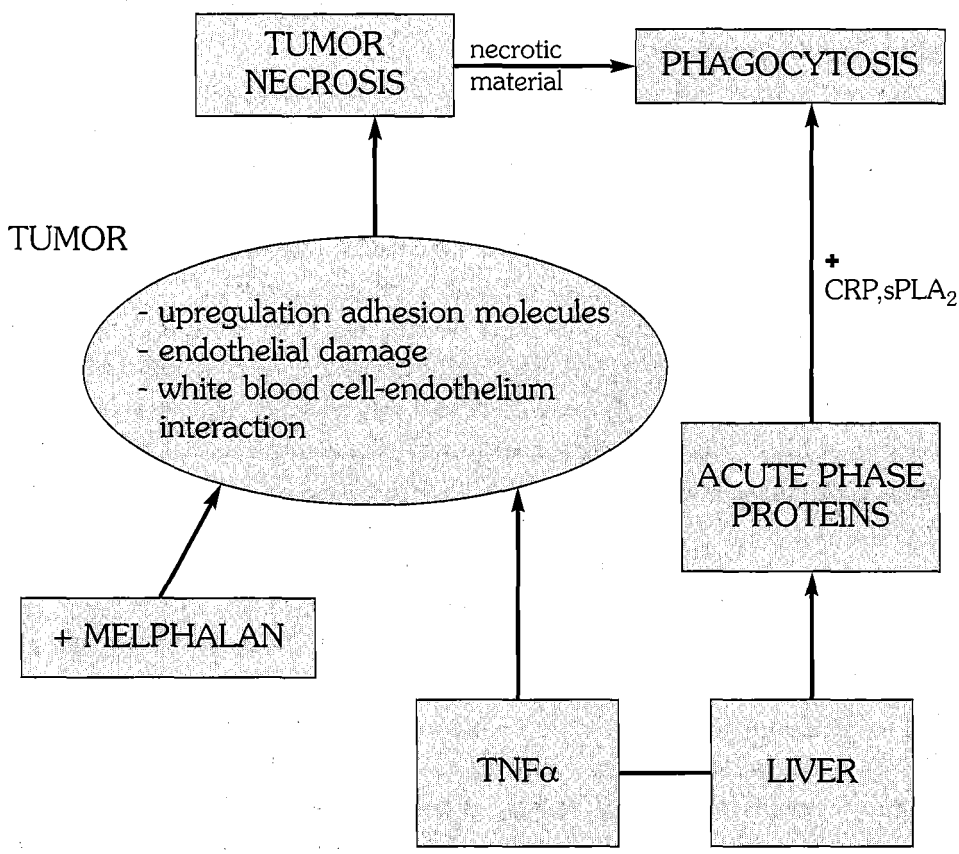


*Figure 1. Schematic representation of the effects of TNFα leading to tumor necrosis, and the interaction with the cytokine- and acute phase response.*

## 7.4 Conclusion

Recombinant TNFα has made a successful return in the clinic because of its application in the setting of isolated limb perfusion. This enabled us to get more insight in the properties of TNFα in humans. We have summarized the current knowledge about the in vitro and in vivo effects of TNFα on monocytes and lymphocytes and the acute phase response, with the emphasis on the effects in humans. In patients treated with intravenous TNFα and in patients undergoing ILP with TNFα and melphalan a rapid and precipitous leucopenia was induced. Differentiation resulted in a rapid decrease of neutrophils and monocytes and a slower decline of lymphocytes and eosinophils. Monocyte- and lymphocyte-function, expressed as ex vivo stimulated cytokine production, decreased within a few hours. Administration of TNFα was followed by a clear acute phase response, probably induced in co-operation with IL-6. Repeated exposure to TNFα resulted in a continuous APR without down-regulation. In figure 1 our findings are represented in a flow diagram.
With the obtained skills and knowledge we ended with a study of the APR in response to administration of IFNα. We observed a preferential increase of ferritin, with only a slight increase or no response of other positive APPs and no response of negative APPs. These findings support our hypothesis that IFNα is implicated in the hyperferritinaemia in adult onset of Still's disease. More study is needed to confirm these findings and to get more insight in the mechanism.

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# CHAPTER 8

## SUMMARY AND CONCLUSIONS

## SUMMARY

During isolated limb perfusion with TNFα and melphalan, patients are exposed to high concentrations of these drugs in the perfused limb and low concentrations in the systemic circulation. The clinical application of this technique, in the treatment of locally advanced melanoma and sarcoma, made it possible to study the immunomodulatory effects of exposure to TNFα and melphalan in man. Effects of longterm exposure to cytokines were investigated in mesothelioma-patients treated with intrapleural TNFα and melanoma-patients treated with subcutaneous IFNα.

In chapter 1 an overview is given of the history of and present developments in the application of TNFα, the acute phase response and treatment with IFNα.
The antitumor effect of TNFα was demonstrated to be mainly mediated by specific destruction of tumor vessels resulting in hemorrhagic necrosis. Clinical application systemic administration of TNFα has been hampered by doselimiting toxicity already at doses too low to mediate antitumor effects. Only with isolated limb perfusion adequate doses of TNFα can be achieved with minimal and easily manageable systemic side-effects. Besides its effects on endothelial cells TNFα is a known stimulator of the acute phase response. In in-vitro studies TNFα was found to induce a specific subgroup of acute phase proteins, called type I acute phase proteins. The in-vivo situation is more complex because of interaction with other cytokines and other mediators. Chapter 1 ends with a paragraph about the interaction of IFNα with other cytokines and acute phase proteins.

In chapter 2 the cytokine-response and effects on monocyte- and lymphocyte number and function were investigated in patients who underwent isolated limb perfusion with TNFα and melphalan. The same parameters were measured in patients after minor and major surgery and in healthy controls. Release of TNFα from the tissues in the perfused limb after ILP resulted in systemic peak levels immediately after ILP, followed by an increase in IL-6. TNFα was not measurable in the other patient groups. IL-6 reached detectable levels during major surgery, but lower peak-levels than in the ILP-patients. No IL-6 could be detected after minor surgery and in healthy controls. The number of monocytes and lymphocytes showed a sharp decline in ILP-patients, which was interpreted as a sign of the margination effect of TNF on leukocytes. In surgical patients and healthy controls no significant change in monocytes or lymphocytes took place. Furthermore

production of cytokines after stimulation in whole blood cell culture (WBCC) was investigated. Both in ILP-patients and major surgery patients cytokine production by stimulated monocytes in WBCC was completely depressed at 3 hours after operation. At day 1 the function was almost restored. In minor surgery patients and in healthy controls cytokine-production did not change significantly. The same pattern was observed for cytokine-production by T-lymphocytes. These results showed a tremendous effect of TNFα on cell-number and cell-function in terms of highly significant differences in cytokine patterns in ILP-patients and also in patients who underwent major surgery.

In chapter 3 the effects of TNFα on number and function of T-cells in ILP-patients were studied in more detail and were further specified. Moreover, effects were differentiated by making a differentiation in T-helper 1 (Th1) and T-helper 2 (Th2) cells. The number of T-cells declined sharply to 5% of the pre-operative value at 3 hours after ILP. Both intracytoplasmic production of cytokines after cell stimulation and cytokine production after stimulation in WBCC showed a decrease in cytokine-levels which started during ILP to hardly measurable levels at 3 hours after ILP. Th1-related cytokines were equally diminished as Th2-related cytokines. In this study no specific relation of TNFα to either Th1-cells or Th2-cells could be demonstrated.

In chapter 4 patients with significant leakage of TNF from the perfusion circuit into the systemic compartment were investigated. Systemic toxicity in these patients was studied in detail after an ILP with leakage to the systemic circulation of 12-65%, and was compared to the clinical course in patients without leakage. Additionally cytokine- and acute phase levels were measured in both groups.
The most prominent clinical toxicity was hypotension, grade III in four patients. This was easily corrected with fluid administration and if necessary dopamine. Hematological toxicity was mild and no renal or pulmonary toxicity was observed. TNFα reached levels up to 277 ng/ml in leakage-patients. Also IL-6 and IL-8 were significantly higher in patients with high systemic leakage. Maximum $sPLA_2$-levels were higher in the leakage-patients whereas levels of CRP were lower, suggestive for higher expenditure of CRP in the removal of injured cells. ILP complicated with high leakage to the systemic circulation is accompanied by manageable toxicity and an elevated cytokine- and acute phase protein response.

After above-mentioned investigation of the inflammatory response of a single exposure to TNFα in chapter 5 the effects of repeated administration of TNFα were examined.

From patients with malignant mesothelioma treated in a phase I trial with intrapleural administration of TNFα every 2 weeks both serum and pleural levels of cytokines and acute phase proteins were determined. The treatment was well tolerated. In pleural fluid administration of TNFα provoked a clear cytokine- and acute phase response with the same pattern after each infusion. In spite of intrapleural TNFα-levels up to 700 ng/ml serum-levels of TNFα and IL-8 remained under detection-level. High IL-6 levels were induced systemically, but with a decreasing response after each intrapleural administration of TNFα. The acute phase response was not diminished: the same pattern of increase, followed by decrease of levels of CRP, $sPLA_2$ and α1-acid glycoprotein remained constant after every administration of TNFα.
Repeated intrapleural administration of TNFα resulted in a clear locoregional and systemic inflammatory response.

The finding that IFNα induced ferritin without elevation of other acute phase proteins, and the resemblance of this patterns with findings in patients with adult onset of Still's disease (AOSD) is elaborated in chapter 6. Patients with high-risk melanoma were treated after excision with subcutaneously administered IFNα 3-5 times a week in a phase III-trial.
Levels of acute phase proteins were determined in serum before treatment and after 4 weeks and half a year during treatment. IFNα induced a significant increase in ferritin-levels without a comparable increase of other positive acute phase proteins. These results confirmed our hypothesis that the high ferritin-levels in patients with AOSD may be a direct effect of IFNα.

In chapter 7 the results of the presented studies are integrated and discussed.

Conclusions made on the basis of the studies are:

- ILP with TNFα and melphalan results in a tremendous decrease in cell-number and function of monocytes and lymphocytes.
- Major surgery has less effect on cell-number but results in a similar almost completely depressed cell-function after operation.
- A specific effect of TNFα on T-helper 1 or T- helper 2 cells can not be demonstrated: both types of T-lymphocytes are completely depressed in number and function after ILP.
- High leakage of TNFα to the systemic circulation during ILP results in manageable toxicity and a clear cytokine- and acute phase response
- Repeated intrapleural administration of TNFα induces a clear locoregional and systemic cytokine- and acute phase response
- IFNα has a direct effect on synthesis or secretion of ferritin, without affecting other acute phase proteins

# SAMENVATTING EN CONCLUSIES

## SAMENVATTING

Tijdens geïsoleerde extremiteitsperfusie met tumor necrose factor-alpha (TNFα) en melfalan worden patiënten blootgesteld aan hoge concentraties van deze middelen in de geperfundeerde extremiteit en lage concentraties in de systemische circulatie. De klinische toepassing van deze techniek, in de behandeling van lokaal vergevorderd (irresectabel) melanoom of sarcoom, maakt het mogelijk om de immuun-modulerende effecten van blootstelling aan TNFα en melfalan in patiënten te bestuderen. De effecten van langdurige blootstelling aan cytokines werd onderzocht in patiënten met een mesothelioom, die werden behandeld met intrapleuraal toegediend TNFα en in patiënten met een melanoom die werden behandeld met subcutaan toegediend interferon-alpha (IFNα).

In hoofdstuk 1 wordt een overzicht gegeven van de geschiedenis van en de huidige ontwikkelingen in de toepassing van TNFα, de acute fase reactie en behandeling met IFNα.
Het anti-tumor effect van TNFα blijkt met name te worden veroorzaakt door specifieke destructie van tumorbloedvaten resulterend in hemorrhagische necrose. Bij klinische toepassing van TNFα wordt systemische toediening van TNFα bemoeilijkt door dosis-limiterende toxiciteit, reeds bij doseringen die te laag zijn om anti-tumor effect te bewerkstelligen. Alleen met geïsoleerde extremiteitsperfusie kan een adequate dosis TNFα bereikt worden met minimale en goed behandelbare systemische bijwerkingen. Naast bovengenoemde effecten op de tumorbloedvaten heeft TNFα een stimulerend effect op de acute fase reactie. In vitro studies laten zien dat TNFα een specifieke subgroep van acute fase eiwitten stimuleert, de zogeheten type I acute fase eiwitten. De situatie in vivo is complexer vanwege interactie met andere cytokines en andere stoffen. Hoofdstuk 1 wordt afgesloten met een paragraaf over de interactie van IFNα met andere cytokines en acute fase eiwitten.

In hoofdstuk 2 worden de cytokine-respons en de effecten op monocyten- en lymfocyten- aantal en -functie bestudeerd in patiënten die zijn behandeld met een geïsoleerde extremiteitsperfusie (ILP) met TNFα en melfalan. In patiënten die een kleine operatie (extremiteit) of een buikoperatie ondergingen en in gezonde personen werden dezelfde parameters bepaald. Afgifte van TNFα uit de weefsels in de geperfundeerde extremiteit na ILP resulteerde in systemische piekwaarden onmiddellijk na ILP, gevolgd

door een stijging in IL-6-waarden. In de andere patiëntgroepen was TNFα niet meetbaar. IL-6 bereikte meetbare waarden tijdens en na abdominale chirurgie, maar lagere piekwaarden dan in de ILP-patiënten. Na een kleine operatie en in gezonde proefpersonen kwam IL-6 niet boven de detectiegrens. Het aantal monocyten en lymfocyten daalde scherp in de perfusie-patiënten. Dit wordt toegeschreven aan het effect van TNFα op de bloedvaten, waardoor leukocyten aan de endotheelcellen hechten. In chirurgische patiënten en gezonde proefpersonen traden geen significante veranderingen op in het aantal monocyten en lymfocyten. Ook de cytokine-produktie na stimulatie in volbloedcelkweek werd onderzocht, als maat voor celfunctie. Zowel in perfusie-patiënten als in de patiënten die abdominale chirurgie ondergingen was 3 uur na de operatie de cytokine-produktie door monocyten na stimulatie in volbloedcelkweek volledig onderdrukt. De volgende ochtend was de cytokine-produktie weer vrijwel geheel hersteld. In patiënten die werden behandeld met een kleine chirurgische ingreep en in de gezonde controle-personen werden geen significante veranderingen gezien in de cytokine-produktie door monocyten. Hetzelfde patroon als hier beschreven voor monocyten werd gevonden in cytokine-produktie door T-lymfocyten. Kort samengevat laat deze studie zien dat TNFα een aanzienlijk effect heeft op cel-aantal en –functie, zich uitend in grote schommelingen in cytokine-produktie in perfusie-patiënten en in patiënten behandeld met abdominale chirurgie.

In hoofdstuk 3 worden de specifieke effecten van TNFα op het aantal en de functie van T-lymfocyten meer in detail beschreven voor een groep patiënten die een geïsoleerde extremiteitsperfusie hebben ondergaan. Daarbij wordt een onderverdeling gemaakt naar effecten van T-helper 1 en T-helper 2 cellen. Het aantal T-cellen daalde snel naar 5% van de pre-operatieve waarde op het tijdstip 3 uur na perfusie. De cytokine-produktie na stimulatie (als maat voor de T-celfunctie) werd op 2 manieren gemeten: in het cytoplasma en in volbloedcelkweek. Beide methoden toonden een scherpe daling in cytokine-produktie, die al startte tijdens de perfusie. Drie uur na perfusie waren er nauwelijks cytokines meetbaar. Er was geen verschil in patroon tussen T-helper 1 en T-helper 2 gerelateerde cytokines. Met dit onderzoek kan dan ook geen specifiek effect van TNFα op T-helper 1 cellen of T-helper 2 cellen worden aangetoond.

In hoofdstuk 4 zijn perfusie-patiënten met significante lekkage van TNFα van de perfusie-circulatie naar de systemische circulatie onderwerp van studie. Systemische toxiciteit in deze patiënten wordt gedetailleerd beschreven. De lekkage-percentages in de onder-

zochte groep patiënten varieerden van 12 tot 65%. De toxiciteit werd vergeleken met het klinische beloop in patiënten die een perfusie ondergingen zonder meetbare lekkage. Ook werd in beide groepen het beloop van cytokines en acute fase eiwitten bepaald.
De meest prominente bijwerking was hypotensie, graad 3 in vier patiënten. Deze lage tensies waren echter vrij eenvoudig te corrigeren met extra infuus en zo nodig dopamine. Hematologische toxiciteit was mild en er was geen nier- of long-gerelateerde toxiciteit. TNFα bereikte waarden tot 277 ng/ml in patiënten met systemische lekkage. Ook IL-6 en IL-8 waren in deze patiënten significant hoger dan in de patiënten zonder lekkage. De maximale waarden van het acute fase eiwit $sPLA_2$ waren hoger in de lekkage-patiënten in vergelijking met de patiënten zonder lekkage. CRP-waarden waren echter lager in de patiëntengroep met lekkage, waarschijnlijk wijzend op een hoger verbruik van CRP bij het opruimen van beschadigde cellen. Concluderend gaat geïsoleerde extremiteitsperfusie met TNFα, gecompliceerd door veel lekkage naar de systemische circulatie, gepaard met behandelbare toxiciteit en een verhoogde cytokine- en acute fase respons.

Na bovengenoemde studies naar de ontstekingsrespons van een eenmalige blootstelling aan TNFα wordt in hoofdstuk 5 het effect van herhaaldelijke toediening van TNFα bestudeerd. In patiënten met een mesothelioom, die werden behandeld in een fase I studie met intrapleurale toediening van TNFα om de week, werden serum en pleurale concentraties van cytokines en acute fase eiwitten bepaald. De behandeling werd goed verdragen. Toediening van TNFα leidde tot een duidelijke cytokine- en acute fase reactie in pleuravocht, met eenzelfde patroon na elke toediening. Ondanks hoge intrapleurale TNFα-concentraties tot 700 ng/ml bleven TNFα en IL-8 in serum onder de detectiegrens. IL-6 bereikte wel meetbare waarden in serum, maar deze waarden werden steeds lager na elke toediening van TNFα. De acute fase respons verminderde niet: hetzelfde patroon van een initiële stijging, gevolgd door een geleidelijke daling, werd gevolgd door CRP, $sPLA_2$ en α1-zure glycoproteïne. Dit patroon bleef zich herhalen na elke toediening van TNFα. Uit deze studie blijkt dat herhaalde toediening van TNFα resulteert in een duidelijke locoregionale en systemische ontstekingsreactie.

In hoofdstuk 6 wordt de waarneming uitgewerkt dat IFNα ferritine induceert zonder stijging in andere acute fase eiwitten. Dit patroon blijkt overeen te komen met bevindingen in patiënten met adult onset of Still's disease (AOSD). Patiënten met een hoog risico melanoom werden na excisie behandeld in een fase III-studie met subcutane toediening van IFNα drie tot vijf keer per week. De concentraties van acute fase eiwitten

werden bepaald in het serum van deze patiënten voorafgaand aan de behandeling, na 4 weken en na een half jaar tijdens behandeling. Behandeling met IFNα induceerde een significante stijging in ferritine-concentraties, zonder een vergelijkbare stijging van andere positieve acute fase eiwitten. Deze resultaten bevestigen onze hypothese dat de hoge ferritine-spiegels in patiënten met AOSD een direct effect van IFNα kan zijn.

In hoofdstuk 7 worden de resultaten van bovengenoemde studies in hun onderlinge samenhang besproken.
Op basis van deze studies kunnen de volgende conclusies worden getrokken:

- Geïsoleerde extremiteitsperfusie met TNFα en melfalan induceert een zeer forse daling van zowel het aantal als de functie van monocyten en lymfocyten.
- Abdominale chirurgie heeft minder effect op het aantal cellen, maar resulteert in een zelfde vrijwel volledig onderdrukte celfunctie na de operatie.
- Een specifiek effect van TNFα op T-helper 1 of T-helper 2 cellen kan niet worden aangetoond: beide typen T-lymfocyten worden qua aantal en functie volledig onderdrukt na geïsoleerde extremiteitsperfusie.
- Veel lekkage van TNFα naar de systemische circulatie tijdens geïsoleerde extremiteitsperfusie gaat gepaard met een behandelbare toxiciteit en een duidelijke cytokine- en acute fase respons.
- Herhaalde intrapleurale toediening van TNFα induceert een duidelijke locoregionale en systemische cytokine- en acute fase respons.
- IFNα heeft een direct effect op de synthese of secretie van ferritine, zonder andere acute fase eiwitten te beïnvloeden.

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## CURRICULUM VITAE

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