Inflammation- and cancer-related cytokine production in immunocompromised hosts. Benefits and risks.

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Summary

Activation of mononuclear cells such as monocytes and macrophages, is the initial step in the host response to bacterial micro-organisms. The host response has the aim to kill the intruders effectively by activating the cellular and humoral components of the innate immunity.
Bacterial products for instance lipopolysaccharides (LPS) activate monocytes and macrophages, which produce and secrete tumor necrosis factor α (TNFα) and interleukin-1β (IL-1β) also called pro-inflammatory cytokines [1-3]. In LPS-mediated signaling lipopolysaccharide-binding protein (LBP), CD14 and the recently described human Toll homolog, TLR4 are essential [4]. When produced in high amounts systemically, TNFα and IL-1β contribute to the deterioration of the patients' hemodynamic condition, resulting in capillary leakage, edema, hypotension, shock and severe tissue damage [2; 3; 5-7]. TNFα and IL-1β can stimulate their own production by positive feedback mechanisms, are capable of inducing the production of other pro-inflammatory cytokines and mediators, such as IL-6, IL-8 and nitric oxide (NO), as well as downregulation by the production of anti-inflammatory cytokines, such as IL-1receptor antagonist (IL-1RA) and IL-10 [8-12].
The produced cytokines (eg.TNFα), bacterial cell wall products and/or activated complement and coagulation cascades leads to endothelial cell activation [13; 14].
During activation of endothelial cells the expression of adhesion molecules is upregulated. Different inflammatory mediators are produced and released by endothelial cells, like IL-6, IL-8 and NO, which exaggerate the initial pro-inflammatory response finally resulting in endothelial damage.
At first, these responses are localized to the site where the infectious agent is present, but when local defense mechanisms are insufficient in eliminating the intruders or when the response is exaggerated, a systemical inflammatory response will be the result. The host response to bacterial intruders (locally and systemically) is the resultant of the continuous balance between pro-and anti-inflammatory mediators [15-17]. In the early phase of an overwhelming systemic infection, an exaggerated pro-inflammatory response dominates, also called systemic inflammatory response syndrome (SIRS). This is followed by a state of hyporesponsiveness, induced by exaggerated amounts of counter-regulatory anti-inflammatory cytokines, such as IL-1RA and IL-10 (compensatory anti-inflammatory response syndrome; CARS). Recent studies support the hypothesis that IL-10 is one of the most important cytokines in inducing CARS; a state of hyporesponsiveness, characterized by high plasma levels of IL-10, a relative low TNFα plasma level and decreased ex vivo production of TNFα upon LPS in whole blood cultures [18-21]. The ongoing decreased HLA-DR expression on circulating monocytes (<30%) and decreased ex vivo TNFα production have predictive value for or are related to fatal outcome [20; 22]. The above described host response to bacterial micro-organisms is an effective mechanism to kill and eradicate bacterial intruders but on the other hand it can be a dangerous cascade when systemically activated, and might lead to severe clinical phenomena like shock, multi-organ failure or even death. SIRS characterizes an abnormal generalized inflammatory

reaction in organs remote from the initial insult. When the process is due to an infection, the terms sepsis and SIRS are synonymous.
From clinical practice it is known that in immunocompromised hosts bacterial micro-organisms can lead to a high morbidity and mortality, higher than in immunocompetent hosts. Different reasons can make a host immunocompromised, such as primary immune deficiencies (congenital or acquired), but also the immaturity of the immune system in newborns, or the impaired immune response in cancer patients treated with chemotherapy.

This thesis deals with **a**. the inflammation-related cytokine responses (in vivo and ex vivo studies) in immunocompromized hosts (newborns and cancer patients) and **b**. leukemia-related spontaneous cytokine responses (in vivo and ex vivo results) which might interfere with inflammation-related cytokine responses when tumors produce high amount of cytokines spontaneously.

In *chapter 2*, we described and used a special laboratory method for intracellular cytokine determinations in monocytes in parallel with the well-known and commonly used enzyme linked immuno sandwich assay technique (Elisa). The kinetics of both intracellular and extracellular accumulation of TNFα and IL-1β in LPS stimulated MNC cultures has been determined. A three-color-immunofluorescence technique was used to detect intracellular accumulation of cytokines. Intracellular accumulation of TNFα in monocytes starts shortly after initiation of the culture; i.e., TNFα is detectable after 1 h, reaching a peak level after 3-4 hours with 50-65% of monocytes staining positive. In parallel with this increased intracellular presence, TNFα was also found in the culture supernatant. The intracellular accumulation of IL-1β in monocytes became detectable after 2 h of culture in the presence of LPS. After 4 h, a plateau was reached, with 90% of the monocytes being positive. In parallel, but with a little delay, IL-1β could be detected in the culture supernatant. TNFα and IL-1β can be produced simultaneously in the same monocytes as was shown by a three-color-immunofluorescence technique. The data demonstrate that **TNFα and IL-1β are good parameters for the early measurement of monocyte activation and that both the intracellular accumulation in monocytes and the amount of secreted cytokines can be used for such a purpose.**

Chapter 3 and 4 describe inflammation related cytokine response in two specific groups of immunocompromised hosts; newborns in *chapter 3* and cancer patients in *chapter 4*.

Newborns are prone to severe bacterial infections due to the immaturity of the immune system, such as lower opsonisation potential due to decreased function of complement, decreased interferon gamma (IFNγ) production by T-lymphocytes and deficient immunoglobulin production by B-lymphocytes [23-26]. In *chapter 3* cytokine responses in neonatal sepsis are described. In chapter 3.1 pro-inflammatory cytokines, such as TNFα, IL-1β and IL-6 are studied during neonatal sepsis with mainly gram-positive bacteria. TNFα and IL-6 plasma levels proved to be

significantly increased in the newborns with sepsis. The IL-1β plasma levels were only slightly elevated in the group newborns with sepsis. After the start of the antibiotics, both TNFα and IL-6 plasma levels decreased concomitantly with the improvement of the clinical situation within 2 days. IL-1β but not the TNFα plasma level appeared to correlate inversely with the decrease in diastolic tension as standardized according to birth weight. TNFα, IL-1β and IL-6 were not correlated with any febrile response in the group with sepsis. We hypothesized that a low IL-1β plasma levels may explain the lack of a febrile response during neonatal sepsis. In conclusion, **TNFα and IL-6 increase many fold at the onset of the invasion with gram-positive micro-organisms in newborns independently of the gestational age.**

In chapter 3.2 and 3.3 we have investigated the possibility of anti-inflammatory cytokine production in vivo during overwhelming bacterial infections in newborns. We hypothesized that low IL-1ra and IL-10 plasma concentrations might contribute to the high morbidity and mortality of neonatal sepsis. IL-1ra and IL-10 plasma concentrations proved to be increased in the newborns with severe infections or sepsis versus the concentrations in the suspected group (IL-1ra) or a control group (IL-1ra and IL-10). We conclude that **the increased morbidity and mortality of neonatal sepsis does not seem to be due to an inadequate IL-1ra and/or IL-10 response.**

The aim of the study, described in *chapter 3.4,* was to examine whether TNFα and IL-6 plasma levels could be of value in diagnosing neonatal sepsis. TNFα and IL-6 were measured in confirmed sepsis, in suspected sepsis and in a control group. When TNFα and IL-6 were combined for the diagnosis of neonatal sepsis, a positive test result for both tests had a sensitivity of 60% and a specificity of 100%. When both tests are positive the diagnosis of neonatal sepsis is almost certain. **The combination of TNFα and IL-6 determinations appears to be a good predictor of neonatal sepsis.**

In cancer patients bacterial infections with severe complications are often seen. Chemotherapy can contribute to an impaired host response to bacterial micro-organisms, like chemotherapy-induced neutropenia, decreased neutrophil function and disturbances of natural barriers. As consequence bacteraemia is associated with a high morbidity and mortality in neutropenic cancer patients. In view of these data the standard care protocols recommend hospital admission and intravenous broad spectrum antibiotic therapy for at least 5 days [27].

In *Chapter 4* the cytokine profiles of cancer patients with fever and chemotherapy-induced neutropenia are studied. Chapter 4.1 deals with the question whether monocytes in ALL patients still play their important role in the host response as in immunocompetent patients. So we determined if the malignancy itself contribute to impaired cytokine profiles in vivo and ex vivo of monocytes. Circulating concentrations of IL-10 and not of TNFα or IL-1β, were elevated at diagnosis of ALL versus at remission. MNC cultures were used to estimate the intrinsic capacity of monocytes to produce TNFα and IL-1β ex vivo. The monocyte number in mononuclear cells at diagnosis of ALL was significantly lower than at remission and

of controls. The number of intracellular TNFα and IL-1β positive cells was positively correlated to the number of monocytes. Also the ex vivo TNFα and IL-1β production in LPS-stimulated MNCs was correlated with the number of monocytes as well as with the number of intracellular TNFα or IL-1β positive cells. But, the ex vivo TNFα and IL-1β production expressed per $1x10^4$ monocytes was nearly equal at diagnosis of ALL, at remission or in controls. Furthermore high IL-10 plasma levels correlated with high ex vivo TNFα production per 10^4 monocytes. These results showed that **monocytes of ALL patients have a normal intrinsic capacity to produce cytokines ex vivo. Monocytes themselves, do not contribute to the impaired host response seen in ALL patients, however, the decreased monocyte number is responsible for the lower TNFα and IL-1β levels ex vivo upon LPS stimulation.**

In chapter 4.2 pro-and anti-inflammatory cytokine profiles (intracellular and in the plasma) in vivo are described to identify diagnostic markers during febrile episodes and define a group of patients at low risk of septicaemia in children with acute leukemia. In this study plasma levels of IL-1β, IL-1ra, TNFα, IL-6, IL-8 and IL-10 were not increased at diagnosis versus remission. At presentation with fever only IL-8 plasma levels were significantly higher in patients with a positive blood culture than in patients with a negative blood culture. The IL-8 plasma levels in both groups are increased compared to IL-8 at diagnosis or at remission. IL-8 levels remained at the same high level for at least 48 hours. We conclude that **plasma IL-8 measurements might be of value as a marker during febrile periods to identify a group with low-risk for sepsis in the future.**

After this study we performed a larger prospective study in which we included patients with fever during chemotherapy-related neutropenia and measured the IL-8, IL-6 and CRP level at presentation (chapter 4.3). The IL-8 and IL-6 plasma concentrations were significantly increased in patients with chemotherapy-related neutropenia and fever due to bacteraemia versus fever of non-bacterial origin. Logistic regression analysis, with sepsis as the outcome variable, revealed significant effects of age combined with either IL-6 or IL-8. No significant effect of leukocyte count, CRP, sex and of underlying malignancy at presentation was detected. The plasma IL-6 and IL-8 levels were strongly correlated with each other. **Using a cut off value with 100% sensitivity, both IL-8 and IL-6 could define a low-risk group for sepsis in neutropenic patients of 28% (95%CI: 15-40%) at start of the febrile period. Intervention studies are warranted to confirm this result and to investigate if an early discharge based on IL-8 or IL-6 measurement is safe, increases the quality of life and results in cost savings.**

Procalcitonin (PCT) is considered as a promising marker, which is sensitive and specific for bacteraemia in intensive care medicine. In cancer patients with fever and neutropenia nothing is known about the value of procalcitonin. Chapter 4.4 compares procalcitonin levels in the group with proven sepsis versus the group with fever of non-bacterial origin (all during chemotherapy-related neutropenia). No differences were found in the plasma levels of procalcitonin between the groups and the PCT levels remained very low with or without proven sepsis. No significant effect of age, sex, underlying malignancy or leukocyte count on PCT could be detected with logistic regression analysis. We suggest that **plasma PCT**

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concentrations seem to have no value in febrile patients with chemotherapy-induced neutropenia, because PCT levels in these patients are low and vary independently of any bacterial infection.

Inflammation-related cytokines could be used as tools in newborns, because no spontaneous cytokine production was seen.
However in cancer patients a number of studies described spontaneous cytokine production in tumor cells in vitro or even reported measurable amounts in vivo [28-34; 35; 36]To be able to interpret the cytokine profiles measured during periods of fever and neutropenia, we were interested in leukemic cells -derived cytokine production. The results are described in *chapter 5*.
We demonstrated that intracellular TNFα, IL-1β, IL-8 and IFNγ are not detectable in pediatric ALL patients, whereas intracellular IL-1β and/or IL-8 was found in 50% and 39% resp. of the 18 pediatric AML patients (de Bont 1996, unpublished data).
The function of AML-derived IL-8 is not well known. Although IL-8 is a potent chemoattractant for neutrophils, more recently, a role of IL-8 in angiogenesis is reported.
Solid tumor growth is dependent on angiogenesis. In AML however, angiogenesis is not well documented.
In chapter 5.1 we addressed the question whether IL-8 produced by AML cells might have a function in angiogenesis. Twelve pediatric patients with acute myeloid leukemia (AML) were selected on the basis of their constitutive in vitro expression of IL-8 by AML cells. IL-8 was detected at protein level (secretion and/or intracellular) and confirmed at mRNA level.
To investigate the role of AML derived IL-8 in angiogenesis, cell free supernatant was collected and used in endothelial cell (EC) migration and proliferation assays. None of the tested AML supernatants induced EC proliferation. In contrast, in the EC migration assay, seven out of the 8 AML supernatants tested, showed an increased EC migration compared to control medium. Moreover, in 6 out of the 8 AML supernatants the increase could be partially blocked by anti-IL-8 antibodies (14% decrease, range 0-37.4%, p=0.036).
In conclusion, spontaneously expressed IL-8 in pediatric AML patients results in in-vitro EC migration and may facilitate an important step in angiogenesis.
In chapter 5.2 twelve bone marrow biopsies of AML patients at diagnosis were studied for vascularity with CD34 staining to visualize endothelial cells. Staining for the presence of FVIII was used when AML blasts were CD34 positive. As controls 5 normal bone marrow samples were used. An increase in vascularity was demonstrated in more than 50% of the AML bone marrow biopsies compared to normal bone marrow. **We concluded that in AML bone marrow biopsies abundant vasculature is present compared to the sparse amount of vessels in bone marrow biopsies of controls. These results suggest that in AML angiogenesis is involved in the ongoing progression as solid tumors for growth.**