Immunomodulatory effects of cardiotrophin-1 on in vitro cytokine production of monocytes & CD4⁺ T-lymphocytes

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Background & objectives: In congestive heart failure (CHF), increased concentrations of several cytokines including cardiotrophin-1 (CT-1) and immunactivation are found. This study was performed to evaluate whether CT-1 can induce in vitro cytokines in monocytes and CD4⁺ T-lymphocytes of healthy volunteers.

Methods: The study was performed in vitro to see whether CT-1 can modulate monocyte or CD4⁺ T-lymphocyte interleukin (IL)-1β, -2, -4, -5, -10, interferon γ (IFNγ), and tumour necrosis factor α (TNFα) expression by flow cytometry following stimulation with CT-1 alone or together with lipopolysaccharide (LPS) or phorbol myristate acetate (PMA)/ionomycine (iono).

Results: CT-1 increased the number of TNFα and IL-1β positive monocytes. LPS induced IL-10, TNFα, and IL-1β in monocytes but only IL-2 in CD4⁺ T-lymphocytes, whereas PMA/iono induced all cytokines besides IL-5 in monocytes and IL-1β in CD4⁺ T-lymphocytes. In LPS activated monocytes, CT-1 induced a concentration-dependent reduction in the number of TNFα positive monocytes. After LPS activation, CT-1 decreased the number of CD4⁺ lymphocytes positive for IL-2, IL-4, and IL-5. In addition, following PMA/iono stimulation, CT-1 initiated a concentration-dependent decrease of CD4⁺ T-lymphocytes positive for TNFα, IL-4, IL-5, and IL-10.

Interpretation & conclusions: The present data show that in vitro CT-1 can activate monocytes and modulate cytokine production of activated CD4⁺ T-lymphocytes. We speculate that CT-1 may at least be partly responsible for immunactivation in CHF.

Key words Cardiotrophin-1 - heart failure - immunactivation

Congestive heart failure (CHF) is a systemic syndrome affecting both the neurohormonal and autonomic nervous systems in addition to displaying immunactivation and haemodynamic effects. The activities of several cytokines are known to be modulated in CHF. Numerous studies have shown increased serum concentrations of proinflammatory cytokines such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1), IL-6, IL-18, and cardiotrophin-1 (CT-1) in CHF.

Monocytes from patients with CHF are characterized by an increased production of TNFα after stimulation with LPS compared to monocytes from healthy controls. Similar results have been
shown in another study reporting that peripheral blood mononuclear cells (PBMC) from patients with CHF produced significantly more TNFα than PBMC from control persons. Further, they found a correlation between levels of TNFα from PBMC and severity of heart failure. However, not only monocyte function was altered in CHF, but also that of CD4+ T-lymphocytes.

T-helper lymphocytes can differentiate into specialized effector cell subsets named Th1 and Th2. Th1 helper cells are responsible for proinflammatory cellular immunity and express IL-2, IL-12, IL-15, IL-18, and interferon γ (IFNγ) while Th2 helper cells express IL-4, IL-5, IL-6, IL-10, and IL-13 as well as mediating humoral immunity. For example, T-lymphocytes of patients with CHF showed increased expression of TNFα, IFN, Fas ligand, IL-10, IL-18, and macrophage inflammatory protein (MIP)-1α in addition to increased surface activation markers such as CD69 and CD25. Fukunaga et al. found Th1 activation in CHF together with a difference between ischaemic cardiomyopathy and idiopathic dilated cardiomyopathy. It is speculated that bacterial translocation and, as a consequence, increased lipopolysaccharide (LPS) concentration in blood because of an impaired gut barrier could be responsible for monocyte/macrophage activation in CHF.

Cardiotrophin-1 is a member of the IL-6 cytokine family that comprises IL-6, IL-11, ciliary neurotrophic factor, cardiotrophin-1, cardiotrophin-like cytokine, leukaemia inhibitory factor (LIF), neuropoietin, oncostatin M, IL-27, IL-11, and macrophage inflammatory protein (MIP)-1α. All these cytokines bind to a specific receptor chain (IL-6R, IL-11R, LIFR). Following cytokine binding, the cytokine/receptor complex associates with glycoprotein 130 (gp130) causing tyrosine phosphorylation of gp130 leading to signal transduction via the Janus kinase (JAK)/signal transducer and activation of transcription 3 (STAT3) pathway. CT-1 is expressed in a time-dependent manner during embryogenesis of organs and in the heart during life. Moreover, it induces cardiac myocyte hypertrophy and is capable of preventing myocyte apoptosis via a mitogen dependent kinase pathway. Increased CT-1 concentrations were detected in patients with acute myocardial infarction and CHF. CT-1 plasma concentration has been shown to correlate with the severity of left ventricular dysfunction. However, in an animal model, CT-1 was found not only to affect myocytes but also the vasculature by decreasing systemic vascular resistance, induction of acute phase proteins in rat hepatocytes, attenuation of endotoxin-induced acute lung injury, and modulation of human umbilical vein endothelial cells and human PBMC.

We hypothesize that CT-1 is able to influence cytokine production in human monocytes and CD4+ T-lymphocytes and, moreover, the cytokine may induce Th1 response in CD4+ T-lymphocytes in vitro. Hence, in this study we investigated the effect of CT-1 on cytokine production in monocytes and CD4+ T-lymphocytes in vitro. The outcome of CT-1 was also analysed on cytokine production of LPS or phorbol myristate acetate (PMA)/iono activated monocytes or CD4+ T-lymphocytes.

**Material & Methods**

**Reagents:** Recombinant human CT-1 was purchased from R & D Systems (Wiesbaden, Germany) and dissolved according to the manufacturer’s instruction. Brefeldin A, LPS from *Escherichia coli* O26:B6, ionomycin and PMA were purchased from Sigma chemicals (Deisenhofen, Germany).

**Subjects:** All subjects (mean age 35.3 ± 7.5 yr) were recruited from the staff of the Department of Internal Medicine I, Jena University Hospital, Friedrich-Schiller-University, Jena, Germany during January - April, 2010. Twelve apparently healthy volunteers (8 females and 4 males) were enrolled in this study. Approval for this study protocol was obtained from the Ethics Committee of the Friedrich-Schiller-University, Jena, and written informed consent were obtained from each subject.

**Sample collection:** Blood samples (9 ml) were collected in lithium heparin tubes from an antecubital vein using a clean venipuncture under controlled venous stasis at 0800 h. The first 2 ml of blood was discharged, and the remaining blood was used for further immediate analysis.

**Cell culture:** Human PBMCs were obtained by Ficoll-paque (Amersham Bioscience, Uppsala, Sweden) centrifugation. The cells were washed three times with PBS, resuspended in RPMI 1640 supplemented with 10 per cent foetal calf serum (FCS) and 1 per cent penicillin and streptomycin (Biochrom AG, Berlin, Germany). All stimulants and media were without significant endotoxin levels according to the manufacturers’ instructions. Pharmacological agents, dissolved in fresh medium, were added to the cells for defined time intervals and concentrations. Fresh medium was added to the cells as a control.
Immunofluorescent flow cytometric analysis of cytokine production: For intracellular staining, peripheral blood was collected in lithium-heparin tubes. Blood (100 μl) was added to RPMI-1640 medium including brefeldin A (final concentration: 1 μg/ml) and incubated for 6 h at 37°C. Next, erythrocytes were lysed by NH4Cl. After washing with PBS/2 per cent FCS, cells were stained with monoclonal antibodies against the surface antigens CD3 (Coulter-Immunotech, Krefeld, Germany) CD4 (Caltag, Hamburg, Germany) and CD8 (BD-Pharomingen, Heidelberg, Germany) (15 min, RT), followed by a washing step and by fixation with 100 μl 2 per cent paraformaldehyde for 10 min at room temperature. After another wash, the cells were incubated in 100 μl permeabilisation solution (0.1% saponin and 0.1% NaN3 in PBS) together with 1 μl directly conjugated anti-cytokine antibodies (IL-1β, IL-2, IL-4, IL-5, IL-10, IFN, and TNFα, all from BD-Pharomingen, Heidelberg, Germany) for 15 min at room temperature. Followed by a further wash with permeabilisation solution, the cells were resuspended in PBS/2 per cent FCS and fluorescence intensity was analyzed by flow cytometry (FACSCalibur, Becton-Dickinson, Heidelberg, Germany). For analysis, regions were defined by forward scatter and side scatter as well as CD3+/CD4- or CD3+/CD8- lymphocyte populations. Data were analyzed with CellQuest Software.

Statistical analysis: Because results were not normally distributed, Wilcoxon test was used to examine the effects of CT-1 and stimulation on cytokine production. Data were analyzed with WinSTAT for Excel. All volunteers were examined only once.

Results

Effect of CT-1 on cytokine expression in PBMC: Stimulation of PBMC with CT-1 revealed a concentration-dependent increase of intracellular TNFα and IL-1β positive staining monocytes (14.8±3.4% positive staining monocytes for TNFα without CT-1 vs. 22.4±4.6% with 100 ng/ml CT-1 equivalent to an increase of 51%, 7.4±2.4% positive staining monocytes for IL-1β without CT-1 vs. 10.6±3.0% with 100 ng/ml CT-1 equivalent to an increase of 43%, Fig. 1). CT-1 did not affect the expression of the other tested cytokines in monocytes and no modulatory effect of CT-1 was observed on the expression of the tested cytokines in CD4+ T-lymphocytes.

Effect of CT-1 in co-stimulation with LPS on cytokine expression in PBMC: Because in severe decompenated heart failure, increased concentrations of endotoxin are...
found\textsuperscript{24}, we tried to imitate this situation in part and stimulated PBMC with LPS (50 ng/ml) together with various concentrations of CT-1 for six hours. This LPS concentration was used based on earlier findings\textsuperscript{25}.

LPS significantly induced TNF\textgreek{a}, IL-1\textgreek{b}, and IL-10 in monocytes but only IL-2 in CD4\textsuperscript{+} T-lymphocytes. Application of LPS together with CT-1 resulted in a CT-1 concentration-dependent decrease of the number TNF\textgreek{a} positive staining monocytes whereas CT-1 had no significant effect on the other tested cytokines under these experimental conditions in monocytes (Fig. 2A). The stimulatory effect of CT-1 alone on TNF\textgreek{a} concentration was much weaker than the effect of LPS on TNF\textgreek{a} expression indicating that CT-1 is only a weak stimulus for the TNF\textgreek{a} expression in monocytes. In CD4\textsuperscript{+} T-lymphocytes, LPS stimulation together with CT-1 resulted in a CT-1 concentration-dependent decrease of IL-2, IL-4, and IL-5 (Fig. 2B).

**Effect of CT-1 in co-stimulation with PMA/iono on cytokine expression in PBMC:** In the next sets of experiments, PMA was used together with ionomycin for PBMC stimulation. PMA/iono caused a significant induction of IFN, TNF\textgreek{a}, IL-2, IL-4, and IL-10 and a significant inhibition of IL-1\textgreek{b} expression in monocytes. CT-1 had no significant effect on the tested cytokines in monocytes.

CD4\textsuperscript{+} T-lymphocytes activated with PMA/iono exhibited a significant increase of the measured cytokines with the exception of IL-1\textgreek{b} (not shown) and IL-5 (Fig. 3). Increasing CT-1 concentrations caused a concentration-dependent decrease of TNF\textgreek{a}, IL-4, IL-5, and IL-10 in activated CD4\textsuperscript{+} T-lymphocytes. The stimulatory effect of PMA/iono was more pronounced compared to the stimulatory effect of LPS on CD4\textsuperscript{+} T-lymphocytes.

**Modulation of Th1/Th2 cytokines by CT-1 in vitro:** The capability of CT-1 to modulate the ratio of IFN/IL-4 in activated CD4\textsuperscript{+} T-lymphocytes in vitro was investigated. Both CT-1 alone and LPS together with CT-1 did not systematically influence the ratio of IFN/IL-4 in CD4\textsuperscript{+} T-lymphocytes (data not shown). PMA/ iono caused an increase of the IFN/IL-4 ratio. In PMA/ iono activated CD4\textsuperscript{+} T-lymphocytes CT-1 caused a concentration-dependent increase of the ratio IFN/IL-4 (Fig. 4). This increase was due to a significant reduction in the number of IL-4\textsuperscript{+} CD4\textsuperscript{+} T-lymphocytes after CT-1 application as CT-1 only slightly and insignificantly decreased the number of IFN\textsuperscript{+} CD4\textsuperscript{+} T-lymphocytes.

**Discussion**

Our study examined **in vitro** cytokine expression of PBMC in healthy volunteers following stimulation with CT-1, a cytokine found increased in CHF which is mainly produced by the failing ventricle\textsuperscript{26}. CT-1 caused a concentration-dependent increase in TNF\textgreek{a} and IL-1\textgreek{b} in monocytes without affecting cytokine production in CD4\textsuperscript{+} T-lymphocytes. Our **in vitro** data are in good agreement with the observation of Conraads et al\textsuperscript{27} who demonstrated that native monocytes from CHF patients produced more IL-1\textgreek{b} and TNF\textgreek{a} than monocytes from healthy controls. Further, cytokine expression increased...
with disease activity in this study\textsuperscript{27}. Although the present results were obtained \textit{in vitro} using PBMC of healthy volunteers, CT-1 caused monocyte activation similar to that found in CHF. Further, \textit{in vitro} CT-1 caused monocyte activation without impairment of the physiologic barrier function of the gut and without bacterial translocation, as well as in the absence of increased serum endotoxin concentrations thought to be responsible for monocyte activation in CHF\textsuperscript{24,27}. Not only monocyte function is modulated in CHF, but enhanced expression of proinflammatory cytokines and activation markers of T-cells have also been reported\textsuperscript{11}. Fukunaga et al\textsuperscript{28} demonstrated that in CHF more CD\textsuperscript{4} T-lymphocytes produce IFN and that the number of CD\textsuperscript{4} IFN producing T-lymphocytes increases with disease activity. The quantity of CD\textsuperscript{4} T-lymphocytes staining for IL-4, however, was not affected significantly\textsuperscript{28}. This group also showed that CHF patients with ischaemic heart disease display a higher proportion of IFN positive CD\textsuperscript{4} T-lymphocytes than patients with idiopathic dilated cardiomyopathy\textsuperscript{12}. In contrast to monocytes, CT-1 alone did not influence cytokine expression of CD\textsuperscript{4} T-lymphocytes. This indicates that CT-1 alone cannot be responsible for CD\textsuperscript{4} T-lymphocyte activation \textit{in vitro}. The exact mechanism of CD\textsuperscript{4} T-lymphocyte activation in CHF is not fully understood and remains to be further elucidated.

Our study also revealed that CT-1 caused a downregulation of several cytokines in activated monocytes and CD\textsuperscript{4} T-lymphocytes. In an animal model of endotoxin-induced lung injury, CT-1 abrogated endotoxin-induced neutrophil accumulation and lung oedema\textsuperscript{23}. Further, CT-1 attenuated endotoxin-induced endothelium-dependent and independent impaired pulmonary vasorelaxation\textsuperscript{23}. Despite decreasing cytokine production in activated monocytes and CD\textsuperscript{4} T-lymphocytes, CT-1 caused a shift to a Th-1 inflammatory pattern in activated CD\textsuperscript{4} T-lymphocytes which could be proven by an increasing IFN/IL-4 ratio. The shift towards a Th-1 inflammatory state in CHF has been previously described\textsuperscript{12}. However, the shift of IFN/IL-4 ratio \textit{in vivo} was driven by the increasing number of IFN producing CD\textsuperscript{4} T-cells whereas in our \textit{in vitro} study, the higher IFN/IL-4 ratio in CD\textsuperscript{4} T-lymphocytes was due to a pronounced decrease in the number of IL-4 producing CD\textsuperscript{4} T-lymphocytes rather than because of an increase of IFN producing T-lymphocytes compared to the quantity of IFN producing CD\textsuperscript{4} T-lymphocytes. Our data suggested that \textit{in vivo} CT-1 might not be responsible for the Th-1 inflammatory shift in CHF.

Regarding study limitations, one constraint of the current study could be the high CT-1 concentrations used compared to concentrations reported in patients with CHF\textsuperscript{17}. Natal et al\textsuperscript{29} found serum CT-1 concentrations of about 100 ng/ml in healthy controls and in patients with metabolic syndrome. Hence, CT-1 serum concentrations in both healthy persons and patients are still a matter of discussion. Yet, independent of the reported CT-1 serum concentration, the amount of CT-1 is likely to be much higher in the myocardium as it is the source of CT-1 in CHF\textsuperscript{26}. Although precise intramyocardial CT-1 concentrations are not known to date, mRNA and immunohistochemical studies showed increased CT-1 expression in hearts of patients with CHF\textsuperscript{30}. Since several proinflammatory cytokines are elevated and PBMC are activated in CHF, it is difficult to study the effect of a single cytokine in PBMC of patients with CHF. For this reason, we employed PBMC from healthy volunteers in culture and stimulated these with recombinant human CT-1. This simplification may explain why all reported changes of cytokine expression in monocytes and CD\textsuperscript{4} T-lymphocytes in CHF could not be determined after stimulation with CT-1. Another reason may be the fact that the expression of cytokines was determined following an incubation period of 6 h. However, there are no reported data for longer incubation periods.

In conclusion, our study demonstrates that \textit{in vitro} the cytokine CT-1 can induce several cytokines in monocytes which are also found increased in CHF. Our data support a new mechanism responsible for monocyte activation in CHF without LPS translocation in the gut. CT-1 may have several modulatory effects on cytokine expression of activated monocytes and activated CD\textsuperscript{4} T-lymphocytes. Because this effect independent of the stimulus was decreased in a concentration-dependent manner by CT-1, it is speculated that elevated CT-1 in CHF may be able to modulate monocyte and CD\textsuperscript{4} T-lymphocyte activation found in CHF.

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