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Down-regulatory effect of *N*-chlorotaurine on tryptophan degradation and neopterin production in human PBMC

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Abstract

N-Chlorotaurine (NCT) plays an important role in the human defense system as a main component of long-lived oxidants, and shows bactericidal, fungicidal, and virucidal activity. Besides this role, NCT seems to act regulatory on immunocompetent cells by altering cytokine production. NCT inhibited nitric oxide, TNF-α, and prostaglandin E_2 (PGE_2) production in activated rodent macrophages, and suppressed superoxide anion, IL-6, and IL-8 formation in human polymorphonuclear leukocytes.

In this study, the influence of NCT on the production of neopterin and the activation of the enzyme indoleamine-2,3 dioxygenase (IDO) was investigated in human peripheral blood mononuclear cells (PBMC). Both events are well established to be triggered by IFN-γ and therefore related to Th1-type immune activation. Mitogen-induced neopterin production as well as tryptophan degradation were drastically reduced upon addition of NCT. Results fit in the concept of a reduction of pro-inflammatory cytokines by this compound. In contrast to earlier results, where NCT was suggested to act primarily down-regulatory on Th2 cells, we propose also a strong suppressive effect of NCT on Th1-type immunity.

Keywords: Taurine chloramine; Interferon-γ; Indoleamine-2,3 dioxygenase; Neopterin

1. Introduction

Human phagocytes are known to use oxidants to attack and eliminate pathogens upon stimulation [1]. Upon phagocytosis, granulocytes and monocytes generate HOCl, which immediately oxidizes NH-groups, forming chloramines [2]. Leukocytes show high intracellular concentrations of free taurine [3], protecting against tissue damage in a variety of inflammatory conditions, such as lung injury [4], ischemic–reperfusion injury [5], rheumatoid arthritis [6], and atherosclerosis [7]. The mechanism of the protective properties of taurine is based on the reaction with HOCl, a strong oxidant, forming the long-lived and less reactive weak oxidant *N*-chlorotaurine (NCT). Another part of the physiological role of NCT is the anti-microbial activity in host defense [8–10]. Further evidence is accumulating that NCT displays regulatory functions on immunocompetent cells. The major part of the studies was performed on activated murine macrophages. In these cells, NCT inhibited the generation of nitric oxide, prostaglandin E_2 (PGE_2), TNF-α, IL-6, and superoxide anion [11–13]. In rat glioma cells, NCT suppressed the production of nitric oxide and PGE_2, monocyte chemoattractant protein-1, and macrophage inflammatory protein-2 production [14,15]. In murine T cells, NCT inhibited IL-2 production in response to mitogens [16], and in activated human leukocytes, NCT suppressed superoxide anion, IL-6, and IL-8 production [17]. A regulatory role of NCT in murine dendritic cells was proposed, based on the modulatory function on IL-6, TNF-α, and IL-12 production in these cells [18].

Due to its presumed anti-inflammatory activity, we investigated the effect of NCT on Th1-type immune response. During Th1-type immune activation, large amounts of

Abbreviations: FACS, fluorescence activated cell sorter; IDO, indoleamine-2,3 dioxygenase; NCT, *N*-chlorotaurine; PBMC, peripheral blood mononuclear cells; ROI, reactive oxygen intermediates

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IFN-γ are produced, acting as a central up-regulator of several effector mechanisms. The pro-inflammatory cytokine is a potent primer for the release of reactive oxygen intermediates (ROI) in macrophages and neutrophils [19]. In parallel, IFN-γ induces the production of neopterin in human monocyte-derived macrophages by activation of GTP-cyclohydrolase I (EC 3.5.4.16) [20]. Recently, human monocyte-derived dendritic cells also were found to produce neopterin derivatives upon stimulation with interferons [21]. In addition, IFN-γ is the main trigger for indoleamine-2,3 dioxygenase (IDO) [22]. This enzyme catalyzes the degradation of the essential amino acid tryptophan to *N*-formylkynurenine, which is subsequently deformylated to yield kynurenine. IDO activity and neopterin formation often correlate very closely due to their common induction by IFN-γ. Both tryptophan degradation by IDO and neopterin production seem to display regulatory function on immunocompetent cells, by induction of apoptosis in T-lymphocytes [23–25]. To investigate the effect of NCT on Th1-type immune response, we tested, in this study, neopterin production and tryptophan degradation in human leukocytes. In addition, a direct effect of NCT on IFN-γ production was investigated.

2. Materials and methods

2.1. Isolation of human PBMC

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood obtained from healthy donors. Separation of blood cells was performed using density centrifugation (Lymphoprep, Nycomed, Oslo, Norway). After isolation, PBMC were washed in phosphate buffered saline containing 1 mM EDTA. Cells were maintained in RPMI-1640 (PAA Laboratories, Linz, Austria), supplemented with 10% heat-inactivated fetal calf serum (Gibco, Invitrogen, Austria), 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2 mM L-glutamine (Serva, Heidelberg, Germany) in a humidified atmosphere containing 5% CO_2.

2.2. Stimulation of PBMC

Isolated PBMC were plated at a density of 3×10^6 cells/ml in supplemented RPMI-1640. Stimulation experiments were performed using the T cell mitogens concanavalin A (Con A; 10 μg/ml) or phytohemagglutinin (PHA; 5 μg/ml; both obtained from Sigma, Vienna, Austria), and co-incubated with 0.05–1.1 mM NCT. Simultaneously, pre-incubation experiments with NCT were performed. Therefore, PBMC were treated with NCT overnight, washed twice with PBS, and then stimulated with Con A or PHA for an additional 48 h. As a control, cells were treated with taurine (0.05–1.1 mM) instead of NCT. For all experiments, pure NCT, as a crystalline sodium salt [26], was dissolved in 0.1 M phosphate buffer (pH 7.2) to 55 mM (1%, stock solution), and adequately diluted in the cell suspensions.

2.3. Measurement of neopterin production, IDO activation, and release of IFN-γ

After incubation, culture supernatants were harvested and frozen until measurement. Neopterin concentrations were determined by ELISA (Brahms, Berlin, Germany). Tryptophan and kynurenine concentrations in supernatants were measured by high performance liquid chromatography (HPLC) as described earlier [27]. IDO activity was estimated by calculating kynurenine to tryptophan ratios (kyn/trp). Production of IFN-γ in culture supernatants of PBMC was measured by ELISA (R&D Systems, Minneapolis, MN, USA).

2.4. Cell viability

Viability of PBMC was assayed after the incubation period by staining with the fluorescing DNA probe propidium iodode. This dye only crosses the membrane of necrotic but not of viable cells. Therefore, cells were harvested after stimulation, resuspended in PBS, and stained with propidium iodide for 15 min on ice. Subsequently, cells were washed and analyzed on a fluorescence-activated cell sorter (FACS). In parallel, cells were counted after staining with trypan blue.

2.5. Statistics

For calculating statistical significances, the Mann–Whitney *U*-test was used. *P*-values below 0.05 were considered to indicate significant differences.

3. Results

3.1. Tryptophan degradation

PBMC treated with NCT (0.05–1.1 mM) showed no significant alterations in tryptophan concentrations (Table 1). In contrast, kynurenine concentrations significantly decreased with higher NCT concentrations (0.28–1.1 mM), leading to a slightly decreased IDO activity as expressed by a diminished kyn/trp ratio (Fig. 1a). Activation of PBMC with Con A or PHA led to a strong decline of tryptophan concentrations (3.7 ± 1.3 and 3.8 ± 1.6 nM tryptophan, respectively), paralleled by an increase of kynurenine levels (9.6 ± 0.9 and 9.7 ± 1 nM). Addition of NCT effectively reduced tryptophan degradation by mitogens in a concentration-dependent manner. A significant reduction of mitogen-induced IDO activity was already observed with 0.05 mM NCT (Fig. 1a). A complete block of mitogen-induced tryptophan degradation was detected when adding 1.1 mM NCT.

In parallel experiments, taurine (0.05–1.1 mM) was added to cell cultures instead of NCT. As shown in Fig. 1b, taurine did not reduce mitogen-induced tryptophan degradation, suggesting the requirement of the *N*-chlorinated species

Table 1
Tryptophan and kynurenine concentrations in culture supernatants of PBMC

NCT (mM)	Control		Con A		PHA	
	Kyn (μM)	Trp (μM)	Kyn (μM)	Trp (μM)	Kyn (μM)	Trp (μM)
–	1.54 ± 0.21	22.8 ± 0.8	9.58 ± 0.86	3.7 ± 1.3	9.72 ± 1.00	3.8 ± 1.6
0.05	1.27 ± 0.43	19.2 ± 2.4	5.20 ± 0.96*	4.4 ± 1.3	6.39 ± 0.75*	1.9 ± 0.6*
0.10	1.19 ± 0.43	19.0 ± 2.1	2.81 ± 0.70*	10.9 ± 1.6*	6.43 ± 0.45*	2.6 ± 0.3
0.28	0.62 ± 0.07*	19.9 ± 1.5	2.59 ± 0.71*	11.9 ± 1.1*	3.21 ± 0.26*	9.7 ± 1.7*
0.50	0.79 ± 0.24*	20.5 ± 1.1	1.48 ± 0.37*	18.0 ± 0.9*	2.63 ± 0.96*	17.4 ± 1.7*
1.10	0.45 ± 0.06*	20.7 ± 0.8	0.55 ± 0.07*	19.1 ± 0.7*	0.50 ± 0.07*	19.4 ± 0.8*

PBMC were incubated with *N*-chlorotaurine (NCT) 0.05–1.1 mM and mitogens concanavalin A (Con A) or phytohemagglutinin (PHA). Control cells were left unstimulated. Tryptophan (Trp) and kynurenine (Kyn) concentrations in culture supernatants were measured after 48 h incubation by HPLC. Values represent mean ± S.E.M.; $n = 6$.

* *P*-values < 0.05.

in this process. To test a possible interaction of NCT with mitogens Con A or PHA, PBMC were pre-treated with NCT for 24 h, washed, and hence stimulated with mitogens. These experiments revealed the same results as those with co-incubation (data not shown).

3.2. Neopterin production

In supernatants of unstimulated human PBMC, an average of 7.2 ± 0.8 nM neopterin was measured after 48 h incubation. Treatment of unstimulated cells with higher

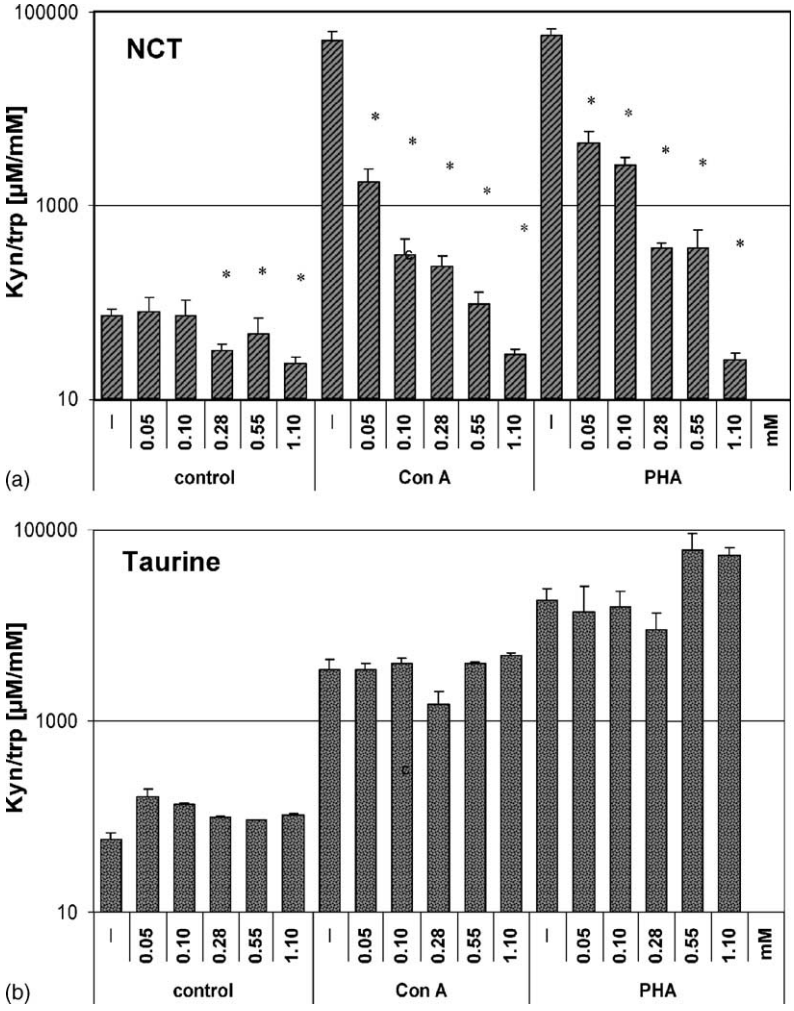

Fig. 1. IDO-mediated tryptophan degradation in response to *N*-chlorotaurine. Human PBMC were stimulated with mitogens concanavalin A (Con A) or phytohemagglutinin (PHA) in the presence of (a) *N*-chlorotaurine or (b) taurine. After 48 h, kynurenine (kyn) and tryptophan (trp) concentrations in culture supernatants were determined by HPLC, and activity of indoleamine-2,3 dioxygenase (IDO) was estimated by calculating kyn/trp. (* *P*-value < 0.01, $n = 6$ in NCT experiments and $n = 3$ in taurine experiments, mention log scale).

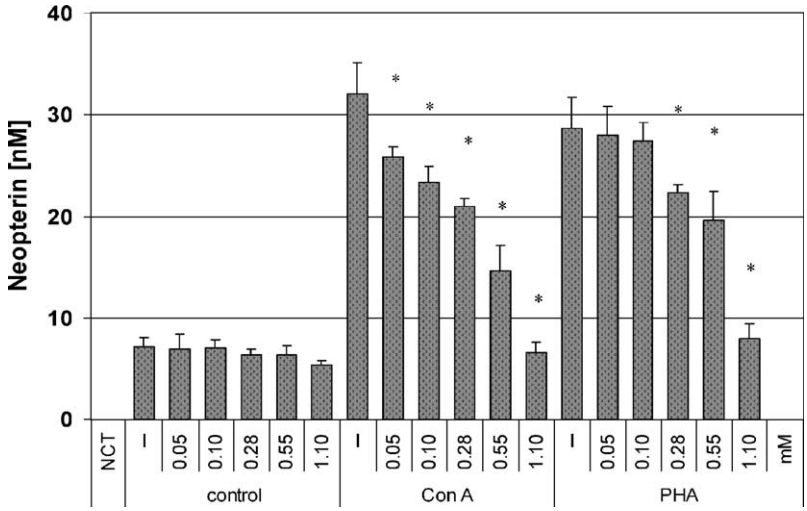

Fig. 2. Influence of *N*-chlorotaurine on neopterin production in PBMC stimulated with concanavalin A (Con A) or phytohemagglutinin (PHA). Freshly isolated human PBMC were stimulated with Con A or PHA with or without NCT. Neopterin production was measured after 48 h incubation in culture supernatants. Bars represent mean ± S.E.M. (* P-value < 0.01, $n = 6$).

concentrations of NCT (0.28–1.1 mM) slightly reduced neopterin production (Fig. 2). After stimulation with the T cell mitogen Con A, a 4.5-fold higher production of neopterin was measured in culture supernatants; PHA induced a four-fold increase in neopterin concentrations as compared to unstimulated control cells. Co-incubation of mitogen-stimulated cells with NCT resulted in a dose-dependent reduction of neopterin formation. The suppression of neopterin formation by NCT was more expressed in cells treated with Con A. Con A-mediated neopterin formation decreased to 80% after addition of 0.05 mM NCT, with 0.1 mM NCT to 73%, with 0.28 mM NCT to 65%, 0.5 mM NCT to 45%, and with 1.1 mM NCT to 20%. In contrast, 0.28 mM NCT was necessary to reduce PHA-mediated neopterin production to 78%, 0.5 mM NCT to 68%, and 1.1 mM to 27%. Again, results from experiments in which cells were co-incubated with NCT and mitogens revealed the same results as those where PBMC were pre-incubated with NCT prior to mitogen incubation. Replacement of NCT by taurine showed no significant reduction in Con A- or PHA-induced neopterin formation (data not shown).

3.3. Release of IFN-γ

In supernatants of PHA-stimulated PBMC, an average of 2.6 ± 0.7 ng/ml IFN-γ was determined after 48 h incubation (Fig. 3). A dose-dependent reduction was observed when stimulated PBMC were co-treated with NCT. The value of IFN-γ determined in supernatants of cells treated with 1.1 mM NCT was comparable with that measured in culture medium of unstimulated PBMC. Similarly, a dose-dependent reduction of IFN-γ production by NCT was observed in Con A-treated cells (data not shown). In contrast, treatment of cells with taurine instead of NCT did not alter

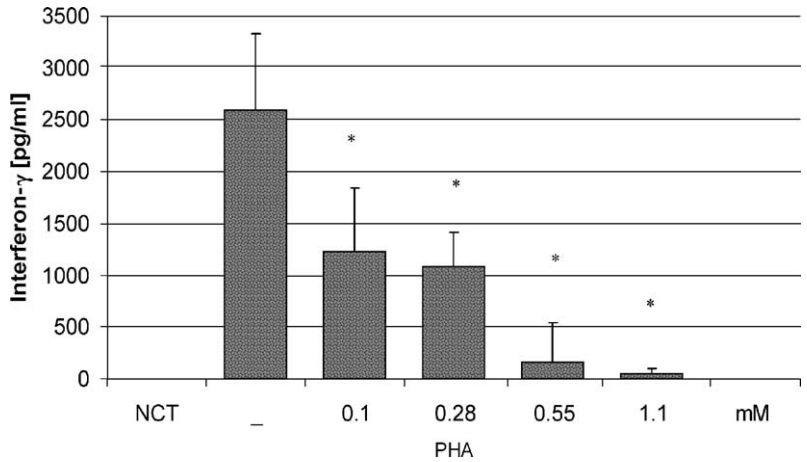

Fig. 3. Release of interferon-γ (IFN-γ) in presence of NCT. PBMC were activated with phytohemagglutinin (PHA) and co-incubated with NCT. IFN-γ in culture supernatants was measured by ELISA. Bars represent mean ± S.E.M. (* P−value < 0.05, $n = 3$).

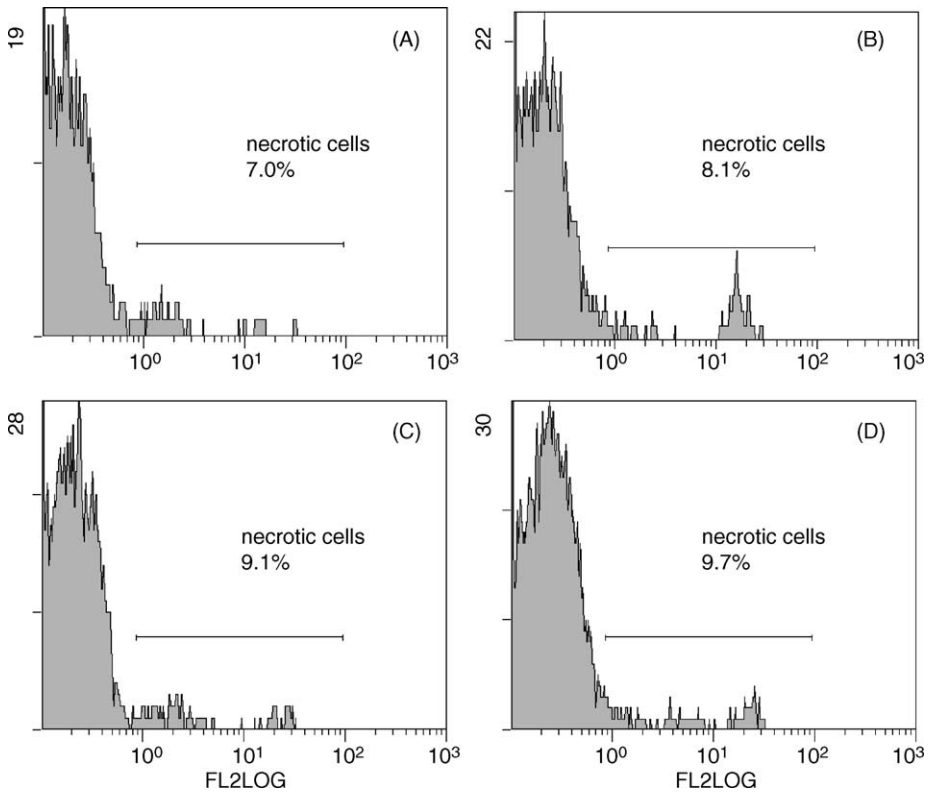

Fig. 4. Cell viability in response to *N*-chlorotaurine. After incubation, PBMC were harvested and stained with propidium iodide to evaluate the amount of necrotic cells. Histogram shows (A) control cells (7% necrotic cells), (B) cells stimulated with Con A (8.1% necrotic cells), and (C) cells treated with NCT 0.55 mM (9.1% necrotic cells) and (D) NCT 1.1 mM (9.7% necrotic cells).

mitogen-induced IFN-γ production in our experiments (data not shown).

3.4. Cell viability

Treatment of PBMC with NCT 0.05–1.1 mM did not alter cell viability in our experiments as shown by propidium iodide assay (Fig. 4). Same results were obtained by the trypan exclusion method (data not shown).

4. Discussion

The in vivo production of NCT depends on activated polymorphonuclear cells, eosinophils, and monocytes producing HOCl. This very reactive compound is bound to taurine, present at high concentrations in the cytosol of leucocytes, forming NCT. This leads to the production of relevant amounts of NCT at the site of inflammation. Besides its role in the detoxification of HOCl and its anti-microbicidal properties, NCT is suggested to act immunomodulatory. In this study, we report that NCT strongly influences biochemical pathways induced during Th1 immune activation, i.e. suppression of IDO activation and reduction of neopterin production via down-regulation of IFN-γ production.

One should be aware that in cell culture media (such as RPMI, which has been used in most of the studies investigating the immune-modulatory effects of NCT), a part of the added NCT chlorinates the amino acids and peptides/proteins of the medium, forming the corresponding *N*-chloro derivatives (transhalogenation) [26]. Although NCT is the most stable *N*-chloro amino acid and therefore remains the highest concentrated active chlorine compound in the system, it cannot be excluded that some of the generated *N*-chloro compounds contribute to the effects seen in this and in former studies. However, since a mixture of chloramines is in any case formed during inflammation in vivo, application of culture media matches the circumstances in the human body, and was suitable for the questions addressed here.

In PBMC, mitogens stimulate a strong activation of the tryptophan-degrading enzyme IDO. Addition of NCT decreased IDO activity in a concentration-dependent manner, whereby pre-incubation of cells with NCT prior to mitogen stimulation showed the same reduction in IDO activity as co-incubation of NCT with mitogen. Therefore, IDO inhibition by NCT is obviously not due to interaction between NCT and mitogens, but rather due to a direct modulation of immune-competent cells by NCT. This observation fits to earlier findings where a direct influence of NCT on intracellular signal transduction pathways was found. In rheumatoid arthritis fibroblast-like synoviocytes, NCT inhibited IL-6 and IL-8 production by diminishing the activity of the redox-sensitive transcription factors NF-κB and AP-1 [28]. Similarly, NCT inhibited iNOS and TNF-α gene expression in activated

rat alveolar macrophages by inhibition of NF-κB activation [29]. Addition of taurine to the PBMC cultures instead of NCT did not alter mitogen-induced IDO activity, demonstrating the requirement of the active chlorine to modulate T cell activity. This finding is in line with earlier findings, showing no effect of taurine on cytokine suppression [16].

A similar effect of NCT on IFN-γ-mediated neopterin production in PBMC cultures was observed. Neopterin formation was strongly down-regulated in the presence of NCT in a concentration-dependent manner, whereas taurine had no influence on this process. Already 0.05 mM NCT was effective to reduce IDO activity in Con A and PHA-stimulated BPMC, as well as neopterin production, in Con A-activated cells. This concentration is below the effective range of NCT in human leukocytes published in earlier studies (0.1–0.4 mM) [30].

Previous data demonstrate that NCT reduces inflammatory cytokine production in murine macrophages, glial cell lines, and in primary cultures of peritoneal macrophages [13–15,31]. In murine dendritic cells, NCT selectively affected the ability of cells to stimulate T-cell activation, suggesting that NCT favors the development of Th1-type rather than Th2-type response [18]. This finding is not supported by our results, showing a strong reduction of IFN-γ production connected with a diminished induction of related biochemical pathways in human PBMC, and thereby a strong inhibitory effect on Th1-type immune response. Neopterin production and activation of IDO are closely linked with Th1-type immune activation [32,33]. One reason for the controversial results might be that our experiments were performed on human and not murine cells. Our suggestion is supported by the earlier observed effect of NCT to suppress lymphocyte proliferation and to reduce not only IL-2, but also IL-6 and IL-8 production in human-activated mononuclear leukocytes [17]. The reduced IFN-γ production upon addition of NCT observed in our study is very likely to be involved in the earlier described reduction of TNF-α elaboration by macrophages [12]. One mechanism of how NCT reduces IFN-γ production might be a reduction of IL-12 production, which was shown in murine dendritic cells [18]. However, also a direct effect of NCT on Th1-type lymphocytes or natural killer cells as major producers of IFN-γ cannot be excluded by our experiments.

The down-regulatory effect of NCT on tryptophan degradation and neopterin production might be of especial clinical relevance in states of chronic inflammation. In this situation, prolonged high levels of IFN-γ induce strongly increased IDO activity and the accumulation of neopterin derivatives. IDO-mediated tryptophan degradation was proposed to slow down T cell activation by inhibition of cell proliferation and induction of apoptosis [23]. Similar effects have been reported from neopterin derivatives [25]. Tryptophan degradation and neopterin production were suggested to be involved in the development of acquired immunodeficiency in the course of chronic immune activation. A reduction of IDO activity and neopterin formation might prevent reactivity of T lymphocytes and impair development of immune deficiency during chronic diseases, thereby assisting the combat against pathogens. Further studies are needed to emphasize this hypothesis.

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