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Effect of neopterin and 7,8-dihydroneopterin on tumor necrosis factor-α induced programmed cell death

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Abstract Tumor necrosis factor-α and the formation of reactive oxygen intermediates are central mediators of apoptosis. Recent data indicated a role of neopterin and 7,8-dihydroneopterin in oxygen radical mediated processes. We have therefore investigated the effect of neopterin-derivatives on TNFα induced apoptosis of the monocyte-like cell line U937. At an elevated concentration 7,8-dihydroneopterin was found to superinduce TNFα mediated programmed cell death due to the formation of reactive oxygen intermediates. Our results imply that in combination with TNFα high concentrations of 7,8-dihydroneopterin enhances apoptosis due to oxidative stress on cells.

Key words: Pteridine; Apoptosis; Reactive oxygen intermediate; Neopterin; 7,8-Dihydroneopterin; Human monocyte

1. Introduction

In response to soluble and particulate stimuli, macrophages secrete a vast number of substances ranging in biologic activity from induction of cell growth to cell death [1]. It is well established that interferon-γ (IFN-γ) upregulates the expression of tumor necrosis factor (TNF) receptors in a variety of cells [2–4] and induces the secretion of neopterin in human macrophages [5,6]. The production of neopterin hereby closely correlates with IFN-γ concentrations [7] and the activation of cell-mediated immunity, e.g. in virus infections including human immunodeficiency virus (HIV) infection, in autoimmune disorders and in certain types of cancer [8–10].

Neopterin is synthesized from guanosine triphosphate (GTP), which is in a first-step enzymatically cleaved by the GTP-cyclohydrolase I to 7,8-dihydroneopterin triphosphate [11]. Since the detection of elevated neopterin and 7,8-dihydroneopterin levels which were found to be secreted in a close correlation of 1:3 in various diseases [12,13], major effort has been put into the clarification of a possible role of neopterin and 7,8-dihydroneopterin in cell-mediated immunity.

Recently, it was demonstrated, that neopterin enhances chloramine-T and hydrogen peroxide mediated chemiluminescence in a luminol assay, while 7,8-dihydroneopterin was shown to be a potent scavenger [14]. Furthermore, neopterin was shown to potentiate toxicity of chloramine-T against bacteria [14]. In contrast, others [15] reported a suppression of superoxide-generating NADPH-oxidase by neopterin in macrophages stimulated with phorbol myristate acetate. Later, Murr et al. [16] illustrated that neopterin enhances hydrogen peroxide effects only in the presence of iron chelator complexes. In a detailed study [17] it was demonstrated that reduced pteridine species were generally potent scavengers, while aromatic pteridines were weak-to-strong enhancers of chloramine-T-dependent chemiluminescence. The confrontation of cells with reactive oxygen intermediates such as hydrogen peroxide, which is also secreted by macrophages, or a depletion of cellular antioxidants may result in programmed cell death (PCD) or apoptosis [18].

It is therefore conceivable that pteridines, in particular neopterin derivatives released by activated macrophages, directly interfere with the cells ability to maintain an appropriate oxidant-antioxidant balance [19]. U937 cells, a human histiocytic lymphoma, undergo apoptosis after incubation with TNFα [20–22]. We have chosen these cells to study the potential effect of neopterin and dihydroneopterin on apoptosis.

2. Materials and methods

2.1. Cell culture

U937 cells, a human histiocytic lymphoma was obtained from ATCC (Rockville, MD). Cells were maintained in RPMI 1640 (Biochrom, Berlin) supplemented with 10% heat-inactivated fetal calf serum (Biochrom, Berlin) and 100 units/ml of penicillin, 0.1 ng/ml of streptomycin and 2 mM L-glutamine (Serva, Heidelberg, Germany) in a humidified atmosphere containing 5% CO_2.

2.2. Chemicals and antibodies used for assays

Human TNFα with a specific activity of 2×10^7 units/mg protein, as assayed on actinomycin D-treated L929 cells by the manufacturer, was obtained from Genzyme (Boston, MA). Propidium iodide staining solution contained 50 μg propidium iodide (Sigma, Vienna, Austria), 0.1% Triton X-100 (Serva, Heidelberg, Germany) and 0.1% tri-sodium citrate-dihydrate (Merck, Darmstadt, Germany) in distilled water. Neopterin and 7,8-dihydroneopterin were purchased from Schircks Lab. (Jona, Switzerland). *N*-Acetylcysteine (NAC) was purchased from Sigma (Munich, Germany) and superoxid dismutase (SOD) from (Serva, Heidelberg, Germany).

2.3. Evaluation of cellular apoptosis

Prior to assays cells were washed once in phosphate buffered saline (PBS; Serva, Heidelberg, Germany) and then plated at a density of 2×10^5 cells per ml complete medium. Cells were preincubated with

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Abbreviations: IFN-γ, interferon-γ; TNFα, tumor necrosis factor-α.

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neopterin derivatives with and without antioxidants (SOD (600 U/ml) and NAC (5 mM) for approximately 2 h and then stimulated with TNFα for one day. Hence cells were centrifuged and resuspended in propidium iodide staining solution and assayed on a FACS Scan (Becton Dickinson, Sunnyvale, CA). Apoptotic cells were characterized by morphology (forward scatter (FSC), versus side scatter (SSC) and by intranuclear contents of fluorescing DNA [23,24].

2.4. Luminol-dependent hydrogen peroxide-induced chemiluminescence determination

Neopterin and 7,8-dihydroneopterin were dissolved in PBS (4.2 mM potassium, 153 mM sodium, 140 mM chloride, 1.47 mM dihydrogen phosphate and 8.1 mM hydrogen phosphate, pH 7.5). A stock solution of 10 mM luminol (Sigma, Munich, Germany) in DMSO (Merck, Darmstadt, Germany) was prepared and freshly diluted to a 40 μM working solution prior to experiments. A mixture of 120 μl of luminol solution (40 μM) and 600 μl PBS containing neopterin or 7,8-dihydroneopterin were preincubated for 1 min in microcuvettes in a luminometer (LKB 1251, Pharmacia, Piscataway, NJ) at 37°C. Hence 140 μl of a 1% hydrogen peroxide solution (0.29 M) were injected into the test tube and light emission was measured for 30 s at a temperature of 37°C.

2.5. Measurement of intracellular generation of reactive oxygen species

Cells were washed three times in HEPES-buffered saline (HBS; 135 mM NaCl, 5 mM KCl, 0.62 mM $MgSO_4$, 1.8 mM $CaCl_2$, 10 mM HEPES, 6.0 mM glucose, pH 7.4) and preincubated with 7,8-dihydroneopterin (5 mM). Hence cells were washed once in HBS, resuspended in HBS containing 1 μg/ml dichlorofluorescein diacetate (DCF; Molecular Probes, Eugene, OR) and plated in quadruplicates at a density of 1×10^5 cells per well in 200 μl in a 96-well plate (Falcon, Becton Dickinson Labware, Lincoln Park, NJ). TNFα was added to cells at a concentration of 1000 U/ml and net generation of reactive oxygen species measured as described [25], in a cytofluor plate reader (Fluoroscan II, Labsystem Genesis, Helsinki, Finland) with an excitation wavelength of 485 nm and an emission wavelength of 538 nm at the indicated time intervals.

3. Results

3.1. The potential role of neopterin and 7,8-dihydroneopterin in the induction of oxidative stress and PCD

U937 cells were preincubated with neopterin and 7,8-dihydroneopterin and activated with recombinant human TNFα. PCD was evaluated by FACS analysis of propidium iodide stained isolated nuclei. Increasing concentrations of neopterin (up to 1 mM, saturated solution) slightly decreased TNFα induced apoptosis as seen in Fig. 1A. In comparison 7,8-dihydroneopterin appeared to inhibit PCD only up to a concentration of 300 μM. Higher doses of 7,8-dihydroneopterin (1 mM) did not alter, and at a concentration of 5 mM even superinduced TNFα mediated apoptosis (Fig. 1B). 7,8-Dihydroneopterin (5 mM) evoked PCD of U937 cells only in combination with TNFα (data not shown here). Additional analysis of cell morphology by FACS analysis of FSC versus SSC and by dUTP-biotin nick end labeling (TUNEL) [24] confirmed apoptosis as a basic mechanism of cytotoxicity (data not shown).

3.2. Effect of neopterin and 7,8-dihydroneopterin on hydrogen peroxide mediated induction of luminol-dependent chemiluminescence

As seen in Fig. 2A and B neopterin and 7,8-dihydroneopterin appeared to scavenge reactive oxygen intermediates and quench hydrogen peroxide induced chemiluminescence. Interestingly, we could observe that high doses of 7,8-dihydroneopterin (5 mM) enhanced the formation of reactive oxygen species (Fig. 2B).

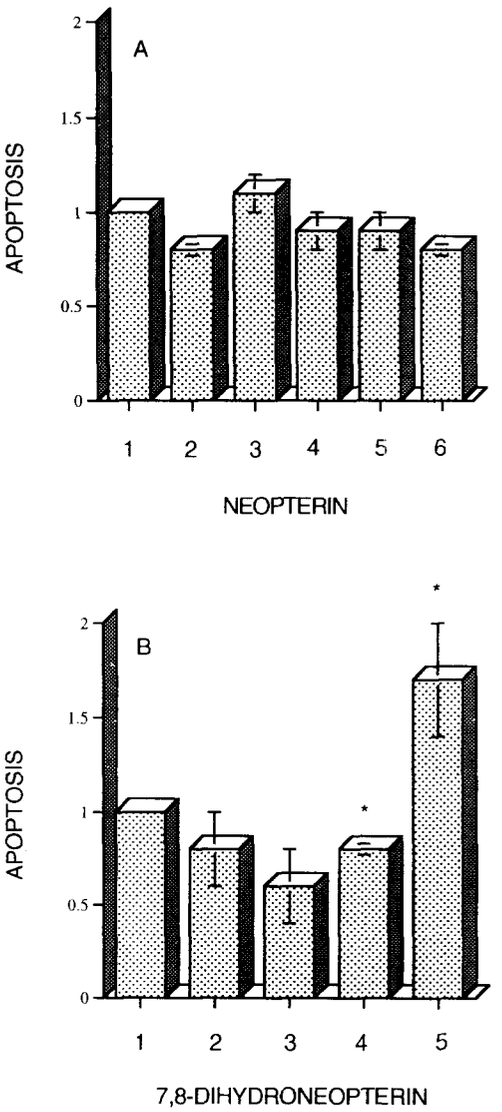

Fig. 1. Effect of neopterin and 7,8-dihydroneopterin on apoptosis of U937 cells. U937 cells were washed once in PBS, preincubated with neopterin (A) or 7,8-dihydroneopterin (B) and stimulated with TNFα, 500 U/ml for 1 day. Apoptotic cells were characterized by measurement of intranuclear contents of fluorescing DNA. Bars show the mean and S.E.M. of 2–7 experiments. Percentage of apoptotic cells in samples pretreated with neopterin and 7,8-dihydroneopterin were divided by percentage apoptotic cells in control (TNFα alone) experiments. (A) Pretreatment of cells with neopterin (1) control, (2) 0.5 μM, (3) 50 μM, (4) 200 μM, (5) 500 μM, (6) 1000 μM. (B) Pretreatment of cells with 7,8-dihydroneopterin (1) control, (2) 10 μM, (3) 200/300 μM, (4) 1000 μM, (5) 5000 μM.

3.3. Effect of antioxidants SOD and NAC on 7,8-dihydroneopterin and TNF induced apoptosis

As shown in Fig. 3 apoptosis induced by TNF alone was not remarkably altered by the addition of SOD. The addition of NAC reduced apoptosis by 16%. In contrast, PCD, mediated by a combination of 7,8-dihydroneopterin (5 mM) and TNFα,

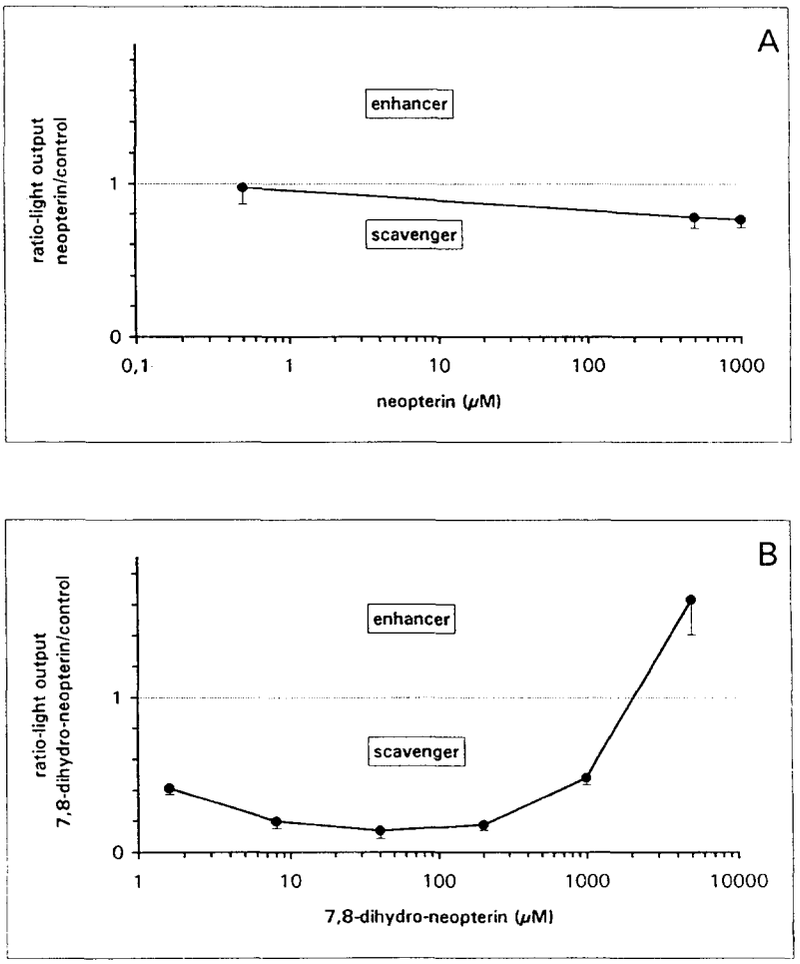

Fig. 2. Effect of neopterin and 7,8-dihydroneopterin on hydrogen peroxide mediated induction of luminol-dependent chemiluminescence. Neopterin and 7,8-dihydroneopterin were dissolved in PBS and incubated with luminol for 1 min at 37°C prior the experiment. Hence hydrogen peroxide (1%) was injected into the test tube and light emission was measured for 30 s at 37°C. (A) Neopterin. (B) 7,8-Dihydroneopterin. The time integral of the light intensity of samples was calculated and divided by that of the control value (sample without neopterin or 7,8-dihydroneopterin). Symbols represent the mean of 3 independent experiments.

was significantly downregulated by SOD (34%) and NAC (47%).

3.4. *Role of reactive oxygen intermediates in 7,8-dihydroneopterin and TNFα mediated apoptosis of U937 cells*

The fluorescent probe dichlorofluorescein diacetate was used to measure the intracellular generation of reactive oxygen species in a cytofluor plate reader. U937 cells were preincubated in the absence or presence of 5 mM 7,8-dihydroneopterin, and

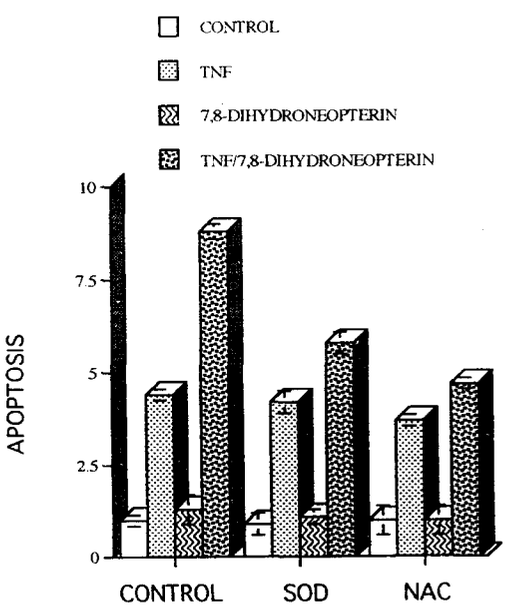

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Fig. 3. Effect of antioxidants, SOD and NAC on 7,8-dihydroneopterin and TNFα induced apoptosis. U937 cells were washed once in PBS, preincubated with or without 7,8-dihydroneopterin and antioxidants (SOD (600 U/ml) and NAC (5 mM) and further stimulated with or without TNFα. Apoptotic cells were characterized by measurement of intranuclear contents of fluorescing DNA by FACS analysis. Percentage of apoptotic cells in samples pretreated with 7,8-dihydroneopterin and TNF were divided by percentage of apoptotic cells in control. Bars show the mean of 2–3 (co-stimulation of TNF and 7,8-dihydroneopterin) experiments.

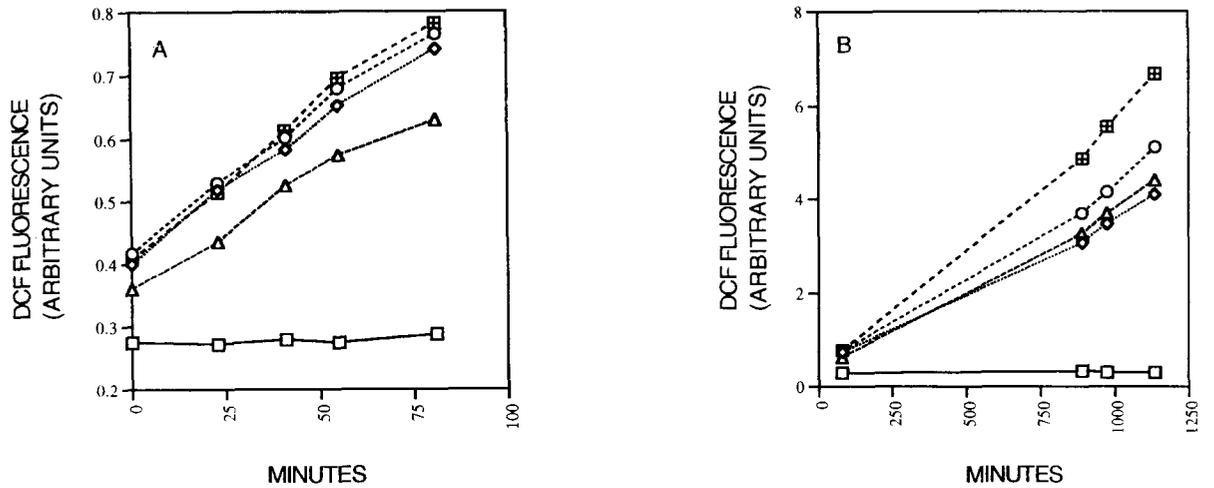

Fig. 4. Measurement of intracellular generation of reactive oxygen species. U937 cells were preincubated with 5 mM 7,8-dihydroneopterin, washed once in HBS and resuspended in HBS containing 1 μg/ml DCF and plated in quadruplicates. TNFα was added to cells at a concentration of 1000 U/ml and net generation of reactive oxygen species measured in a cytofluor plate reader with an excitation wavelength of 485 nm and an emission wave length of 538 nm. (A) Short time interval (1) 0, (2) 23, (3) 41, (4) 55, (5) 81 min. (B) Long time interval (1) 891, (2) 976, (3) 1136 min. Values are representative of the means of quadruplicates. □, U937 without DCF; ◇, control; , TNF; △, dihydroneopterin; ⊞, dihydroneopterin + TNF.

further activated with TNFα. As seen in Fig. 4, cells continuously produced reactive oxygen throughout the experiment. During the first time interval of 1.4 h when cells were monitored in the cytofluor plate-reader, 7,8-dihydroneopterin appeared to scavenge reactive oxygen species (Fig. 4A). 15 h later, the net cellular generation of reactive oxygen species was not any more diminished in cells preincubated with 7,8-dihydroneopterin but was slightly augmented like in TNF treated U937 cells (Fig. 4B). Over the time period of 19 h reactive oxygen intermediates continuously increased due to a combined treatment of cells with 7,8-dihydroneopterin and TNF (Fig. 4A and B).

4. Discussion

IFN-γ upregulates the expression of TNF receptors [2–4] and induces the secretion of neopterin and 7,8-dihydroneopterin [5,6]. The production of neopterin and 7,8-dihydroneopterin, closely correlates with IFN-γ concentrations and the activation of cell-mediated immunity, e.g in HIV infection, levels of neopterin, IFN-γ and soluble TNF receptor increase in parallel with progression of the disease [8,26].

The data presented here provide evidence that in accordance with TNFα [18], neopterin and 7,8-dihydroneopterin may intervene with the ability of a cell to maintain an appropriate oxidant–antioxidant balance. High doses of 7,8-dihydroneopterin superinduce TNFα-mediated apoptosis. U937 cells were preincubated with neopterin and 7,8-dihydroneopterin, and PCD was evaluated by FACS analysis of propidium iodide stained isolated nuclei [23,24]. Increasing concentrations of neopterin (up to 1 mM) and 7,8-dihydroneopterin (up to 300 μM) decreased TNF-induced apoptosis. Higher doses of 7,8-dihydroneopterin (1 mM) did not alter, and at a concentration of 5mM even superinduced TNFα mediated apoptosis.

Contradictory opinions exist in the literature regarding the relationship of reactive oxygen intermediates and neopterin. One study [14] showed, that neopterin enhances hydrogen peroxide mediated chemiluminescence in a luminol assay, while others [15] reported a suppression of superoxide-generating NADPH-oxidase by neopterin in macrophages stimulated with phorbol myristate acetate. Later [16] it was illustrated, that neopterin enhances hydrogen peroxide effects only in the presence of iron chelator complexes. In the present study we have observed that increasing concentrations of neopterin scavenge reactive oxygen intermediates in a luminol-dependent chemiluminescence assay. In accordance with previous data [27,14] we could demonstrate, that increasing concentrations of 7,8-dihydroneopterin scavenge reactive oxygen intermediates and quench hydrogen peroxide induced chemiluminescence. Interestingly, we discovered that high doses of 7,8-dihydroneopterin (5 mM) enhanced the formation of reactive oxygen species.

Effects of neopterin and 7,8-dihydroneopterin on TNFα induced apoptosis of U937 cells may therefore be better understood. Neopterin up to 1 mM, and lower doses of 7,8-dihydroneopterin act as scavengers of reactive oxygen intermediates and therefore inhibit TNF mediated apoptosis. A high dosis of 7,8-dihydroneopterin (5 mM), however, in combination with TNF apparently leads to increased formation of reactive oxygen intermediates and therefore superinduced apoptosis. This observation is in accordance with data in the literature [28] that reduced pteridines react with molecular oxygen under formation of free radicals.

Eukaryotic cells produce reactive oxygen as products of phagocytic activity or cellular respiration and are continuously required to balance oxidants and anti-oxidants. Above normal levels of reactive oxygen intermediates are referred to as oxidative stress and may result in apoptosis [29,30,18]. Oxidative stress-associated PCD can be blocked by compounds with antioxidant abilities, e.g. TNFα mediated PCD [20] can be inhibited by NAC, a thiol antioxidant and glutathione precursor [31,32] or by increased levels of SOD, a class of metalloproteins that catalyze the dismutation of superoxide-radical to molecular oxygen and hydrogen peroxide [33]. In our study PCD mediated by a combination of 7,8-dihydroneopterin (5 mM) and TNFα was significantly downregulated by SOD and NAC.

Previous findings could be confirmed by measuring the intracellular generation of reactive oxygen species in a cytofluor plate-reader using the fluorescent probe dichlorofluorescein diacetate [25]. U937 cells were preincubated with or without 5 mM 7,8-dihydroneopterin and further activated with TNFα. Cells continuously produced reactive oxygen throughout the experiment. During the first time interval of 1.4 h, 7,8-dihydroneopterin alone appeared to scavenge reactive oxygen species. 15 h later, net cellular generation of reactive oxygen species was not any more diminished in cells preincubated with 7,8-dihydroneopterin, but in accordance with results found in cells treated with TNFα alone was slightly augmented. Over the time period of 19 h reactive oxygen intermediates continuously increased in cells treated with a combination of 7,8-dihydroneopterin and TNF.

Pteridines obviously interlude with the cells potential to balance reactive oxygen intermediates. Increasing concentrations of neopterin (up to 1 mM) and 7,8-dihydroneopterin (up to 300 μM) reduce reactive oxygen species and decrease TNFα induced PCD. High concentrations of 7,8-dihydroneopterin and TNFα induce unphysiological levels of oxygen radicals which induce oxidative stress on cells leading to apoptosis. High levels of antigenic and cytokine activity results in the production of reactive oxygen intermediates. In response TNF is produced, likely providing an amplification loop for the induction of free radical activity [34,30]. It is possible to conceive, that high concentrations of neopterin/7,8-dihydroneopterin, which, e.g. are common in HIV stand in direct context with defective T-cell responses in patients [35]. Based on our findings high doses of 7,8-dihydroneopterin may superinduce TNFα-mediated apoptosis of T-cells and contribute to the deficiency of cell-mediated immunity.

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