# Effect of a Histone Deacetylases Inhibitor of IL-18 and TNF-Alpha Secretion in Vitro

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## Abstract

**BACKGROUND:** Interleukin-18 (IL-18) and Tumor Necrosis Factor-alpha (TNF-α) are proinflammatory cytokines that increased the development of Th1 immune response, but have a different type of regulation of the gene expression. Whereas TNF-α has an inducible expression, IL-18 is translated as an inactive protein and required proteolytic cleavage by Casp-1 in inflammasome complexes.

**AIM:** To investigate the effect of the histone deacetylases inhibitor Suberoylanilide Hydroxamic Acid (SAHA) on the gene expression and secretion of both cytokines, IL-18 and TNF-α, according to their contribution to the cancer development and anticancer immunity.

**METHODS:** Isolated peripheral blood mononuclear cells (PBMC) were stimulated with LPS and C3bgp with or without SAHA. Cytokine production was assessed by ELISA at 6 and 24h.

**RESULTS:** IL-18 and TNF-α secretion was significantly increased at 6h and 24h in response to stimulation. TNF-α production from stimulated PBMC was downregulated by SAHA at 6 and 24h. Treatment with SAHA does not inhibit the secretion of IL-18 significantly either at 6 or 24h of stimulation.

**CONCLUSION:** The inhibition of histone deacetylases by SAHA does not influence the inflammasome-dependent production of immunologically active IL-18. In contrast, the production of proinflammatory TNF-α in cultures was mediated by the activity of HDAC class I and class II enzymes.

## Introduction

The inducible cytokine release is regulated at several levels started from chromatin remodelling allowing gene expression and finalised with protein secretion. Before transcription, the gene region must be accessible to the transcriptional factors binding. The key role in this process has the histone modification mainly acetylation and deacetylation. Two classes of enzymes drive the acetylation status of the chromatin. Acetyltransferases open the chromatin conformation by histone acetylation and allow the transcriptional process. Histone deacetylases (HDACs) compress the chromatin structure triggering the gene silence by deacetylation [1][2]. HDACs are divided into four classes. Of them, class I are resident to the nucleus, where they act as histone modifiers and repressors of the transcription. Histone deacetylases class II is moving between the nucleus and cytoplasm. They can regulate the gene expression also by the changes in acetylation/deacetylation status of other proteins [3][4].

Recently, a new class of small organic molecules-HDAC inhibitors (HDI), which abolish the action of HDACs, are intensively studied, especially about cancer and inflammatory diseases treatment [5][6]. They regulate the expression up to 10% of the cellular genes by affecting enzymes including in chromatin remodelling complex and recruiting of the transcription factors [7]. Although the histone acetylation is linked to an increased transcription; HDI also can increase the expression of some genes by the still unclear mechanism. Suberoylanilide Hydroxamic Acid (SAHA) is the HDI interacting with

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class I and class II histone deacetylases [4]. SAHA was the first HDI approved by U. S. Food and Drug Administration for the treatment of some malignant disease as CTCL [8].

During the early phase of the immune response, binding of microbial antigens (especially those referring to pathogen-associated molecular patterns-PAMPs) to pattern recognition receptors (PRR) of the immune cells, activate intracellular signalling pathways, which in turn lead to alteration in cell behaviour and gene expression. As a consequence soluble mediators are synthesised and secreted by the activated immune cells including proinflammatory and immunoregulatory cytokines like TNF-α and IL-18. Tumor necrosis factor-alpha (TNF-α) was first discovered as mediating cell death of some malignant cells. Recently, a growing body of evidence showed that TNF-α has a tumour-promoting role as a key mediator of chronic inflammation which drives the cancer development [9]. TNF-α is secreted as a soluble 17-kDa molecule after processing of membrane-bound TNF-α by constitutive expressed membrane TNF-α converting enzyme [10], mainly by the activated macrophages/monocytes. Interleukin-18 (IL-18) is also a proinflammatory cytokine, but it is included in Th1 polarisation. It's inducible gene expression after recognition of PAMPs by PRR lead to the synthesis of an inactive protein (pro – IL-18). Pro – IL-18 is converted into biologically active IL-18 by another activation pathway in inflammasome complexes through caspase 1-mediated cleavage [11]. IL-18 is involved in the development of successful antitumor immunity through its ability to induce IFN-γ secretion [12]. Unlike TNF-α, the mechanisms regulating IL-18 processing and secretion remains not well understood.

In this regard, our study was designed to investigate the SAHA effect on protein synthesis and release of TNF-α and IL-18 from stimulated healthy human PBMC.

## Methods

### Isolation of PBMC

Peripheral venous blood was taken by venipuncture from 10 healthy donors after the approval of the Ethics Board of Medical Faculty, Trakia University. Each volunteer was informed and signed informed consent. The samples (10 ml) were collected in sterile tubes with EDTA. Peripheral blood mononuclear cells (PBMC) were harvested after density gradient centrifugation over Histopaque-1077.

### In vitro culturing

PBMC (1 x $10^6$ cells/ml) cultures were prepared as described previously by Dobreva et al. [13]. They were stimulated with: 30 µg/ml C3 binding glycoprotein, (C3bgp) [14]; or 1 µg/ml Lipopolysaccharide (LPS) from Escherichia coli (Sigma-Aldrich-Merck, Darmstadt, Germany). PBMC cultures were incubated at $37^oC$ for 6 and 24h. After incubation the separated supernatants were stored at $-70^oC$.

### HDAC inhibition

SAHA (Sigma-Aldrich-Merck, Darmstadt, Germany) (5 µM) was used for the inhibition of histone deacetylases. The inhibitor was added one h before stimulation.

### Cytokine evaluation

Assessment of IL-18 and TNF-α was performed by ELISA, under to the manufacturer's instructions. For the detection of TNF-α, R&D Systems Qantikine ELISA kit (Minneapolis, MN 55413, USA) was used. IL-18 production was measured using commercially available kits purchased from MBL International Corporation (Woburn, MA 01801, USA). The colour reaction was measured as OD units and expressed in pg/ml. The sensitivity of the ELISA kits was 12 pg/ml for IL-18 and 15 pg/ml for TNF-α.

### Statistical analysis

The data was presented as means and standard error of the mean. Evaluation of the statistical differences between cultures was performed by Student's t-test. Differences were significant when the P value was equal or less than 0.05.

## Results

### TNF-α production was suppressed by SAHA

Results presented in Figure 1 demonstrated that C3bgp and LPS increased significantly TNF-α production at 6 and 24 h in comparison with nonstimulated controls (p < 0.05). The addition of SAHA to stimulated cultures leads to significantly decreased TNF-α production at 6 and in higher degree at 24 h. TNF-α quantity secreted by PBMC cultured with HDAC inhibitor was 4 to 6-fold less than in cultured PBMC without SAHA at six h and 5 to 8 fold less at 24 h.
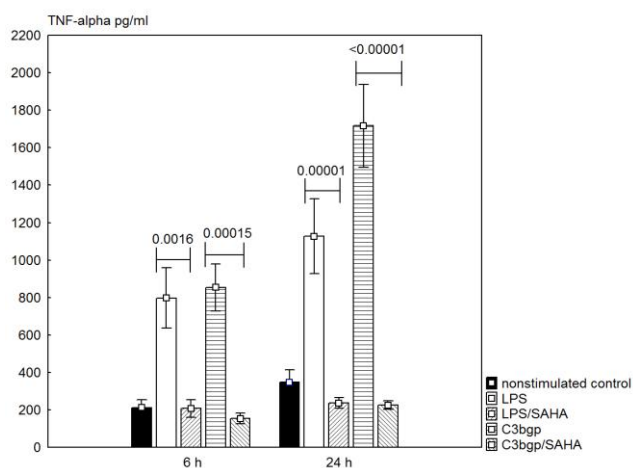
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_Figure 1: TNF-α production from stimulated with LPS and C3bgp PBMC after inhibition of HDAC by five µM SAHA at 6 and 24 h. P value between stimulated cultures without SAHA and stimulated cultures with SAHA is indicated on the figure_

### SAHA did not modulate significantly IL-18 production

The addition of C3bgp and LPS leads to significantly more IL-18 production in comparison with nonstimulated cultures ($p < 0.05$). Moreover, we did not observe significant differences between 6 and 24 h in the secretion of IL-18 from stimulated cultures. The inhibition of HDAC slightly decreased IL-18 production. However, we did not detect significant differences between stimulated cultures treated with SAHA and cultures without SAHA as shown in Figure 2.
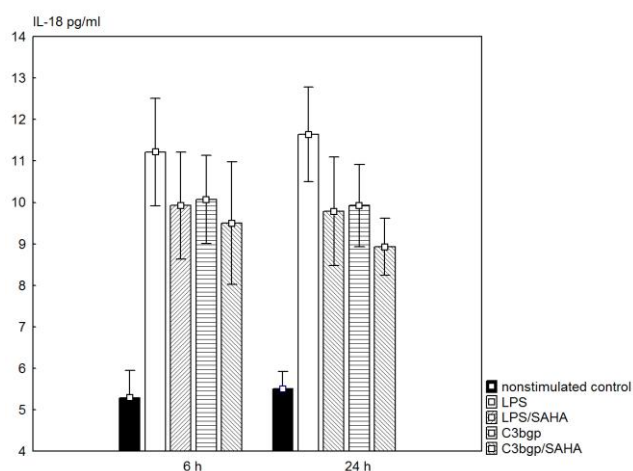


_Figure 2: IL-18 production from stimulated with LPS and C3bgp PBMC after inhibition of HDAC by five µM SAHA at 6 and 24h_

## Discussion

IL-18 and TNF-α are proinflammatory cytokines, with a different effect on the acquired immunity and different production and regulatory mechanism as well. Whereas TNF-α gene expression and secretion is mediated through the TLR signalling pathway, IL-18 required proteolytic processing by Casp-1 in inflammasome before its secretion [11]. In the current study, we evaluated the effect of the histone deacetylases inhibitor SAHA on the gene expression and secretion of both cytokines, IL-18 and TNF-α, according to their contribution to the cancer development and anticancer immunity.

TNF-α is a main proinflammatory cytokine identified for the first time because of its rapid cytolytic effect on some experimental cancers [15]. Recently, new studies showed that TNF-α has protumorigenic activity and is involved in all key points of tumorigenesis – tumour promotion, malignant transformation, tumour cell proliferation, angiogenesis and malignant cell spreading [9]. This investigation showed that the addition of the HDAC inhibitor SAHA downregulated the production of TNF-α released by the stimulated mononuclear cells. The same results were obtained from other authors [16]. Today is accepted that histone deacetylation is linked to the decreased gene transcription. Nevertheless, there was experimental evidence demonstrating that the treatment with HDI downregulated the expression of some proinflammatory cytokines by a mechanism which currently is being studied extensively. For example, Takada et al. demonstrated that HDI SAHA did not affect the binding of NF-kB transcription factor to the promotors of the target genes, but inhibited IkBα kinase activation, IkBα phosphorylation and translocation of p65 to the nucleus [17]. It is widely accepted that TNF-α production in response to LPS is mediated by TLR4 followed by the activation of NF-kB. Our study demonstrated that SAHA downregulated TNF-α production after stimulation with LPS. Therefore, our results are in accordance with decisions of the other investigators [17][18] that the downregulating effect of SAHA on the proinflammatory cytokine production is mediated by the suppression of NF-kB transduction pathway. Previously we showed that C3bgp activated JNK and p38 intracellular transduction pathways [19]. Furthermore, our results showing that SAHA inhibited C3bgp-mediated TNF-α production are in concordance with the study of Ajizian et al., demonstrating that the suppression of p38 MAPK leads to downregulated TNF-α production [20] and with the study of Choo and coauthors, showing that SAHA affected p38 activation [21].

Currently, it is widely accepted that IL-18 drives the Th1 immune response, because induces IFN-γ secretion from T cells and natural killer cells [12]. There is evidence that treatment with IL-18 of experimental animals has significant antitumor action [11]. Moreover, in vivo IL-18 administration in experimental mice inoculated with tumour cell line stimulated IFN-γ production and IL-12 independent antitumor response [22]. Its elevated levels have been

observed in several types of cancers, especially in advanced cancers with metastasis [23][24][25]. Our study indicated that SAHA did not influence significantly IL-18 production from PBMC. There are few studies about SAHA and its effect on IL-18 secretion. However, our results contradict the study of Choo et al., which demonstrated that SAHA inhibits the production of IL-18 in E11 and THP-1 cell lines in a dose-dependent manner [26]. One explanation for this discrepancy may be the different cell sources of IL-18. Our experiments were done with PBMC from healthy donors. In their study Choo et al., used E11 cell line of human rheumatoid synovial cells, transformed with simian virus 40 large T antigen expression vector or a THP-1 monocytic cell line derived from a patient with acute myeloid leukaemia. Moreover, it is well known that the tumour cell lines had specific regulation of the gene expression in comparison with normal human cells [27].

Recently, it is widely accepted that chronic inflammation has a crucial role in the tumour-promoting and survival, regardless of its origin. In this process, proinflammatory cytokines play a key role [28]. Many studies indicated that SAHA suppresses proinflammatory cytokines like IL-12, IFN-γ, TNF-α and IL-1β expression [13][16]. However, not always the inhibition of IL-12 and IFN-γ has positive effects, because of their immunoregulatory function as cytokines triggering cell-mediated anticancer immunity. Our study has shown that treatment with SAHA leads to decreased TNF-α and unmodified IL-18 production by PBMC-a surprising effect on the synthesis and secretion of both cytokines. Considering the protumorigenic activity of TNF-α, the downregulation of its production is a desirable effect of treatment with various histone deacetylase inhibitors including SAHA. However, IL-18 like IL-12 has antitumorigenic activity because of its property to induce Th1 cell-mediated type of the immune response. Therefore, under immunosuppression triggered by SAHA, an unaffected IL-18 production may have a crucial role in the realization of antitumor immunity. Thus, this SAHA-specific modulation of the TNF-α and IL-18 production may provide a further clinical advantage for the treatment of various malignant conditions.

In summary, our study showed that HDAC inhibitor SAHA downregulated TNF-α production and did not affect IL-18 secretion from activated PBMC. Therefore, we conclude that the production of TNF-α is mediated by HDAC class I and class II enzymes, but they are not involved in inflammasome-dependent regulation of biologically active IL-18 secretion. This different regulation of both cytokines by histone deacetylases and their inhibitors may be used in the development a new approach in the therapy of certain types of cancers.

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