Research Article

Cornelia A. Karg, Rebecca Lisandrelli, Günther Schider, Pablo Monfort-Lanzas, Anita Siller, Harald Schennach, Simone Moser, Dietmar Fuchs, Johanna M. Gostner*

Extracellular spermidine attenuates tryptophan breakdown in mitogen-stimulated peripheral human mononuclear blood cells

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Abstract: Polyamines, particularly spermidine, play a vital role in regulating cellular functions and influencing processes such as proliferation, ageing, and immune modulation. This study explores the effects of spermidine on the immunoregulatory pathway of tryptophan breakdown, which is mediated by indoleamine 2,3-dioxygenase 1 (IDO-1), as well as on neopterin synthesis, in peripheral blood mononuclear cells. Spermidine treatment was found to suppress IDO-1 activity in mitogen-stimulated cells in a dose-dependent manner without affecting neopterin synthesis. This suppressive effect on tryptophan breakdown contributes to the debate surrounding the health-promoting antioxidant properties of spermidine. However, the divergent regulation of the two immunobiochemical pathways investigated, which share common upstream signalling events, requires further exploration.

Keywords: spermidine, tryptophan, indoleamine 2,3-dioxygenase, immunoregulation, neopterin

*** Corresponding author: Johanna M. Gostner**, Institute of Medical Biochemistry, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria, e-mail: johanna.gostner@i-med.ac.at
Cornelia A. Karg: Institute of Medical Biochemistry, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria; Institute of Pharmacy, Department of Pharmacognosy, University of Innsbruck, Innsbruck, 6020, Austria
Rebecca Lisandrelli, **Pablo Monfort-Lanzas:** Institute of Medical Biochemistry, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria; Institute of Bioinformatics, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria
Günther Schider: Institute of Medical Biochemistry, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria
Anita Siller, **Harald Schennach:** Central Institute of Blood Transfusion and Immunology, University Hospital Innsbruck, Tirol Kliniken GmbH, Innsbruck, 6020, Austria
Simone Moser: Institute of Pharmacy, Department of Pharmacognosy, University of Innsbruck, Innsbruck, 6020, Austria
Dietmar Fuchs: Institute of Biological Chemistry, Biocenter, Medical University Innsbruck, Innsbruck, 6020, Austria

Abbreviations

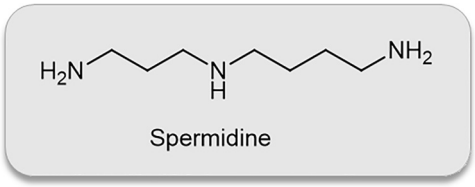
GTP-CH-I	guanosine triphosphate cyclohydrolase 1
IDO	indoleamine 2,3-dioxygenase
IFN-γ	interferon gamma
Kyn	kynurenine
ODC	ornithine decarboxylase
PBMCs	peripheral blood mononuclear cells
PHA	phytohaemagglutinin
ROS	reactive oxygen species
SAM	*S*-adenosylmethionine
TDO	tryptophan 2,3-dioxygenase
Trp	tryptophan

1 Introduction

Polyamines are small aliphatic polycations that are present in all types of living cells. In mammals, the main polyamines, spermidine (Scheme 1), spermine, and their precursor putrescine, are synthesized from ornithine and methionine via decarboxylation. They regulate processes, including cell growth and proliferation, transcription and translation, development, ageing, carcinogenesis, and immune regulation [1,2].

Intracellular polyamine levels are tightly regulated through their biosynthesis, catabolism, and transport since polyamines are essential, but excessive concentrations lead to toxicity due to the degradation to toxic metabolites such as aldehydes, ammonia, peroxides, and acrolein. Ornithine decarboxylase (ODC) catalyzes the rate-limiting step, converting ornithine to putrescine, which is then transformed into spermidine and spermine [1].

The regulation of ODC is critical, being stimulated by growth-promoting factors such as hormones, mitogens, and carcinogens, while elevated intracellular polyamines trigger multiple negative feedback mechanisms [3,4]. The diet-derived vitamin folic acid plays an important role,



Scheme 1: Structure of spermidine.

which fuels one-carbon metabolism, enabling methionine/*S*-adenosylmethionine (SAM) synthesis and thereby connects methylation to polyamine production and cell growth.

Altered polyamine levels are linked to diseases [5–7]. Decades ago, it was shown that polyamine levels are elevated in the urine of cancer patients and, later on, several studies confirmed the presence of increased concentrations in cancer tissue and other body fluids [8,9]. Recent findings report similar alterations in lung, pancreatic, and prostate cancers [10–12]. Polyamines support neoplastic proliferation and are being explored both as biomarkers (e.g. for colon cancer) and as targets for anticancer therapy, with their elevated levels in rapidly growing tissues and upregulation by growth-promoting hormones further underscoring their role in cell proliferation [13–18]. Conversely, they also contribute to stress defense, as oxidative stress induces the transcription of ODC and spermidine/spermine-*N*1-acetyltransferase and increases ODC activity [19,20].

Polyamines exhibit multifaceted roles in the regulation of the immune response, and early studies predominantly attributed them to immunosuppressive effects [6]. A variety of anti-inflammatory effects have been described, e.g. by directly interfering with Janus kinase (JAK) signaling [21]. Additional anti-inflammatory properties attributed to spermidine are the inhibition of lipid peroxidation, the increased production of anti-inflammatory cytokines [22,23], as well as the decreased formation of nitric oxide and the downregulation of interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) in lipopolysaccharide (LPS)-stimulated mouse macrophages [24]. In LPS-stimulated microglia cells, spermidine reduced the production of nitric oxide and prostaglandin E2, which was associated with the downregulation of the expression of inducible nitric oxide synthase and cyclooxygenase-2 [25]. However, it has become apparent that the role of polyamines in immunoregulation is highly cell-type and condition-specific [26] and the molecular mechanisms involved include both covalent binding, site-specific, orthosteric and allosteric interactions, and non-specific interactions [21,26,27].

During cellular immune activation, neopterin production via guanosine triphosphate-cyclohydrolase I (GTP-CH-I) and tryptophan degradation by indoleamine 2,3-dioxygenase 1 (IDO-1) are strongly induced, with interferon gamma (IFN-γ) being the most important activating cytokine [28]. IDO-1 is the rate-limiting enzyme in the oxidative breakdown of tryptophan (Trp) to kynurenine (Kyn) via the intermediate *N*-formyl-kynurenine. Trp is an essential amino acid, which serves as a building block for proteins and the precursor of the neurotransmitter 5-hydroxytryptamine (serotonin). The IDO-1-mediated breakdown of Trp is a critical immunoregulatory pathway, whereby both the reduction of Trp and the formation of bioactive Kyn downstream metabolites need to be considered. In a variety of disorders that go along with chronic immune activation, elevated blood kynurenine to tryptophan ratios (Kyn/Trp) have been observed [29]. Blood Kyn/Trp is used as an index of the IDO-1 activity when accompanied by an increase of immune activation markers such as neopterin [28].

Peripheral blood mononuclear cells (PBMCs) are powerful primary human cell model that allow the investigation of immunomodulatory properties of compounds, taking the monocyte/T cell crosstalk into account. PBMC stimulation with the mitogen phytohaemagglutinin (PHA) has been used previously to characterize the effects of a variety of small molecules on IDO-1 activity and neopterin formation [30,31].

In this study, PHA-stimulated PBMCs were used to investigate the immunomodulatory activity of the polyamine spermidine on the biochemical pathways of Trp breakdown via IDO-1 and neopterin formation via GTP-CH-I as readout [32].

2 Materials and methods

2.1 Isolation of PBMCs

Human PBMCs were isolated from anonymized residual whole blood, provided by the Central Institute of Blood Transfusion and Immunology (University Hospital, Innsbruck, Austria), and collected from healthy blood donors who had agreed to the use of residual blood for scientific research. The study adhered to the principles of the Helsinki Declaration, and the local ethics committee confirmed that no further approval was required for the use of anonymized leftover samples from the local blood bank for research purposes. PBMCs were freshly isolated prior to each experiment using density gradient centrifugation with Lymphoprep separation medium, as described earlier [31]. Briefly, 15 mL of whole blood was diluted with an equal volume of heparinized phosphate-buffered saline

(PBS). The lymphocyte separation medium (10 mL, PAN Biotech, Aidenbach, Germany) was carefully covered with a layer of diluted blood. After centrifugation for 40 min at 4°C at 1,000 rpm, the PBMC layer was harvested. Cells were washed three times with PBS-EDTA and stored in RPMI 1640 medium supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% (v/v) 200 mmol/L L-glutamine (both from GIBCO, Thermo Fisher Scientific, Germany), and gentamicin (50 µg/mL) under standard cell culture conditions.

2.2 Spermidine solutions

A PBS-EDTA buffer stock of 137.70 mmol/L spermidine (Sigma-Aldrich, Vienna, Austria) was prepared, sterile-filtered, and stored in aliquots at −20°C. In the experiments, spermidine was diluted in culture medium at concentrations ranging from 0.31 to 10 µmol/L.

2.3 Cell viability

Cell viability of PBMCs was assessed in both PHA-stimulated and unstimulated cells after 48 h of incubation with increasing concentrations of spermidine, including controls using the CellTiter-Blue® assay kit (Promega, Mannheim, Germany), which measures cell metabolic activity by the reduction of resazurin to resorufin. Fluorescence of resorufin was measured after 4 h of incubation in a humidified atmosphere containing 5% CO_2 at 37°C in the dark, with excitation at 560 nm and emission at 590 nm, using a Tecan Infinite F200 PRO plate reader.

2.4 PBMC treatment and metabolite measurements

PBMCs were seeded in 48-well plates at a density of 1×10^7 cells/mL in supplemented culture medium and incubated with increasing concentrations of spermidine. After 30 min of treatment, 20 µL of the medium (unstimulated cells) or PHA (stimulated cells, final concentration 10 µg/mL) was added, and cells were incubated for 48 h in a humidified atmosphere containing 5% CO_2 at 37°C in the dark. An aliquot of the cell suspension was centrifuged at 13,000 rpm for 10 min, and 100 µL of the resulting supernatant was mixed with 100 µL of the internal standard (25 µmol/L 3-nitro-L-tyrosine) and 25 µL of 2 mol/L trichloroacetic acid (Carl Roth, Karlsruhe, Germany) to precipitate proteins. After two additional centrifugation steps at 13,000 rpm for 10 min each, the supernatant was analyzed for Kyn and Trp concentrations by reverse-phase HPLC using a LiChrosorb C18 column (5 µm particle size, Merck, Darmstadt, Germany) and isocratic elution with 15 mmol/L acetic acid–sodium acetate (pH = 4.0), following the method of Widner et al. with minor modifications [33,34]. The system used was a Varian ProStar HPLC system equipped with a solvent delivery module (model 210), an autosampler (model 400, Varian ProStar), an UV-spectrometric detector (SPD-6A, Shimadzu), and a fluorescence detector (model 360, Varian ProStar). Kyn and 3-nitro-L-tyrosine concentrations were determined by a UV detector at a wavelength of 360 nm. Trp was detected by its natural fluorescence at an excitation wavelength of 286 nm and an emission wavelength of 366 nm. Varian Star Chromatography Workstation (version 6.30) software was used.

Neopterin concentrations were determined by enzyme-linked immunosorbent assay (ELISA) (BRAHMS, Hennigsdorf/Berlin, Germany) according to the manufacturer's instructions with a detection limit of 2 nmol/L [33].

2.5 Bioinformatics analysis

Spermidine target genes were identified from the drug signatures database (DSigDB) [35]. Functional annotation of these genes was performed using ClueGO [36] for Gene Ontology Biological Processes (GO BP), and disease with the DOSE R package [37]. Transcription factor (TF) enrichment analysis was conducted using EnrichR [38] with the TRRUST Transcription Factors 2019 database. Finally, spermidine-predicted TFs were compared with upstream TFs of IDO1 from the hTFtarget database [39].

2.6 Statistical analysis

The half-maximal inhibitory concentrations (IC50) were calculated based on the method of Chou and Talalay using CalcuSyn software (Biosoft, UK; Version 1.1.1) [40]. Values were extrapolated from the viability data of the three highest doses. If the viability exceeded 100%, it was adjusted to 99.9%. Statistical analysis was performed using the IBM SPSS Statistics Version 21 (SPSS, Chicago, USA). The average of the technical replicates of each independent experiment was used to calculate the SEM. Due to the small

sample size (n = 3–4), non-parametric tests (Friedman and/or Wilcoxon signed-rank test) were used. p-values below 0.05 were considered to indicate statistically significant differences.

Ethical approval: The research related to human use has been complied with all the relevant national regulations, institutional policies and in accordance with the tenets of the Helsinki Declaration, and has been approved by the authors' institutional review board or equivalent committee.

Informed consent: Informed consent has been obtained from all individuals included in this study.

3 Results

3.1 Effects of PHA stimulation and spermidine treatment on PBMCs

The potential cytotoxic effects of spermidine treatment were assessed in either unstimulated or PHA-stimulated PBMCs after 48 h of incubation time. Stimulation of PBMCs with the mitogen PHA at a concentration of 10 µg/mL alone led to a slight increase in metabolic activity assessed by resazurin conversion, although the difference was not statistically significant (Figure 1a). These findings provide further evidence supporting previous reports on the stimulatory effects of PHA on cellular proliferation [24]. Treatment with increasing concentrations of spermidine did not affect cell viability within a concentration range of 0.3–2.5 µmol/L. A reduction in cell viability was observed at 5 µmol/L only in PHA-stimulated cells and only at the highest concentration of 10 µmol/L in unstimulated and PHA-stimulated cells (Figure 1b).

Estimated IC50 values were 15.76 µmol/L (extrapolated from the highest three doses, 95% CI: 11.77–21.10 µmol/L) in stimulated and 20.08 µmol/L (extrapolated from the highest three doses, 95% CI: 19.54–20.64 µmol/L) in unstimulated cells.

3.2 Effects of PHA stimulation on Trp, Kyn, and neopterin concentrations in PBMCs

The effect of spermidine treatment on Trp to Kyn breakdown was assessed in the supernatants of both unstimulated and PHA-stimulated PBMC via HPLC analysis. After an incubation period of 48 h, the average concentration of Trp in the supernatants of unstimulated cells was measured to be 34.5 ± 1.2 µmol/L (mean ± SEM). This corresponds to 93.2 ± 9.04% of the initial medium content, which was 37 µmol/L. Concurrently, the mean concentration of Kyn was found to be 0.9 ± 0.1 µmol/L, resulting in a Kyn/Trp ratio of 25.5 ± 2.4 µmol/mmol. Upon stimulation with PHA, the Trp content in the supernatants decreased significantly to 16.7 ± 0.7 µmol/L, corresponding to only 45.2 ± 5.03% of the initial medium content. In contrast, the mean concentration of Kyn increased substantially to 9.4 ± 0.4 µmol/L, resulting in a Kyn/Trp ratio of 573.6 ± 45.3 µmol/mmol. The concentration of neopterin in unstimulated cells was measured to be

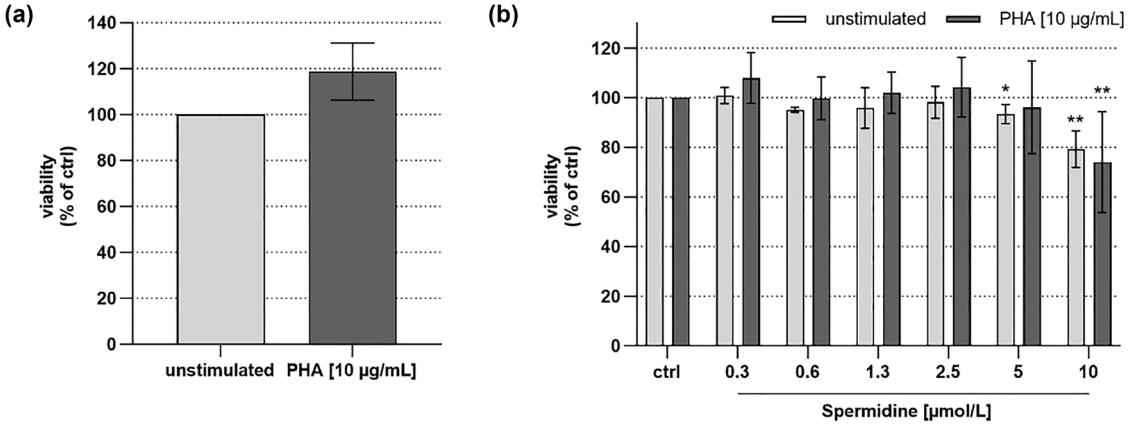


Figure 1: (a) Viability of unstimulated (light grey bars) and PHA-stimulated (dark grey bars) PBMCs after cultivation for 48 h. (b) Effect of increasing concentrations of spermidine on cell viability of PBMCs after 48 h of treatment. Cell viability was tested using a CellTiter-Blue® assay, which assesses the metabolic activity indicated by resazurin conversion. Results are expressed as % of control cells (*p < 0.05, **p < 0.01, compared to respective vehicle control). The results shown are mean ± SEM based on four independent experiments run in technical triplicate.

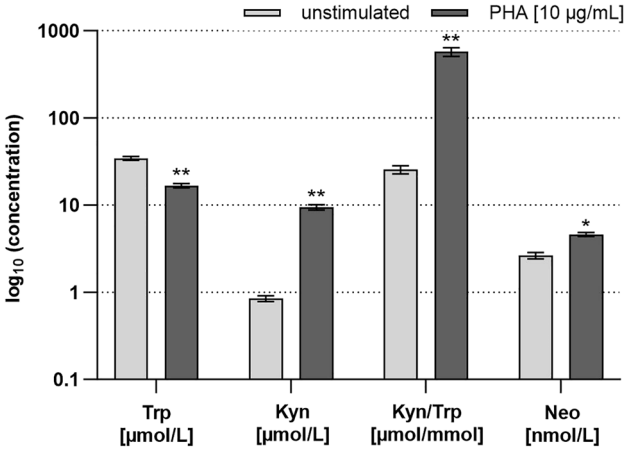


Figure 2: Comparison of the Trp, Kyn, Kyn/Trp, and neopterin (Neo) concentrations in unstimulated and PHA-stimulated PBMCs. Supernatants of unstimulated (light grey bars) and PHA-stimulated PBMCs (dark grey bars) were analyzed via HPLC or ELISA for their Trp concentrations (µmol/L), Kyn concentrations (µmol/L), Kyn to Trp ratio (µmol/mmol), and neopterin (nmol/L) concentrations (*p < 0.05, **p < 0.01, each compared to unstimulated cells). The results shown are mean ± SEM based on four independent experiments (four different donors) for Trp and Kyn, and three independent experiments for neopterin, each experiment was run in duplicate.

2.6 ± 0.2 nmol/L, and upon stimulation, it increased to 4.6 ± 0.2 nmol/L (Figure 2).

3.3 Effects of spermidine on Trp, Kyn, and neopterin concentrations in PBMCs

In unstimulated cells, treatment with spermidine had no significant effect on Trp degradation or neopterin formation. In contrast, spermidine significantly increased Trp concentrations in PHA-stimulated cells, particularly in the range of 2.5–10 µmol/L. The Trp concentration increased by about 35% compared to the corresponding vehicle control. This increase was accompanied by a significant reduction in Kyn levels, which decreased in the same concentration range to 21% as compared to the control values. The activity of IDO-1 was estimated by calculating the Kyn to Trp ratio (expressed as µmol Kyn/mmol Trp) and turned out to be significantly reduced, reaching 11.8% of the control ratio after treatment with 10 µmol/L spermidine. Therefore, these results demonstrate that

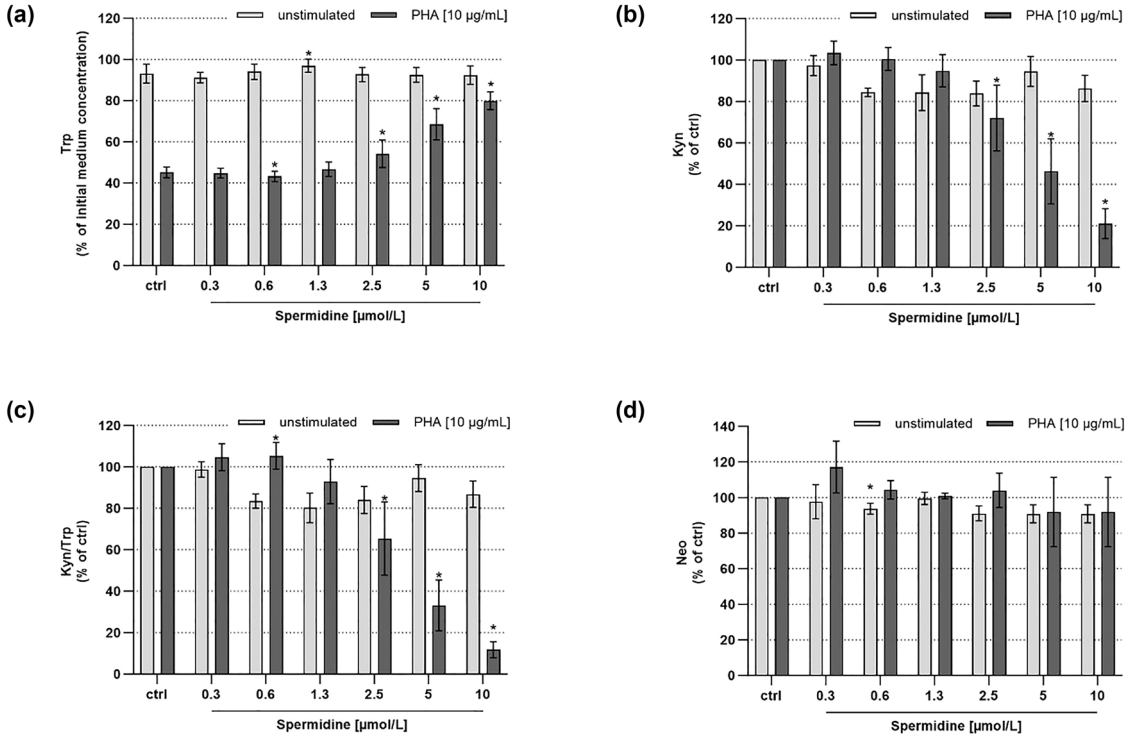


Figure 3: Effect of spermidine on (a) Trp and (b) Kyn concentrations, (c) Kyn to Trp ratio, and (d) Neo concentrations in unstimulated (light grey bars) and PHA-stimulated (dark grey bars) PBMCs after treatment for 48 h. Metabolite concentrations were analyzed via HPLC or ELISA for Neo. Data are expressed as percentages of the medium control (*p < 0.05, **p < 0.01, compared to respective vehicle control), presented as mean ± SEM, based on four independent experiments (four different donors), each performed in duplicates.

spermidine exhibits a dose-dependent inhibitory effect on IDO-1. Remarkably, neopterin concentrations remained unchanged (Figure 3).

3.4 Functional analysis of spermidine target genes

To further characterize the effects of spermidine, 45 spermidine-target genes were identified from public databases. Functional annotation revealed significant enrichment in pathways associated with ROS metabolism, including "Response to reactive oxygen species" and "Cellular response to oxygen-containing compounds." Additional enriched pathways included "Response to organic cyclic compound," "Positive regulation of transport," and "Cellular amine metabolic process" within the Gene Ontology Biological Processes (GO BP) category (Figure 4a).

Next, the diseases associated with spermidine-target genes were analyzed, identifying 50 diseases and conditions. The top five diseases enriched in this analysis were primarily linked to respiratory conditions, cardiovascular disorders, and liver diseases (Figure 4b). To explore potential regulatory mechanisms affected by spermidine, TF enrichment analysis was performed (Figure 4c). A total of 15 TFs were identified as regulators of multiple spermidine-target genes. To establish a link between spermidine's

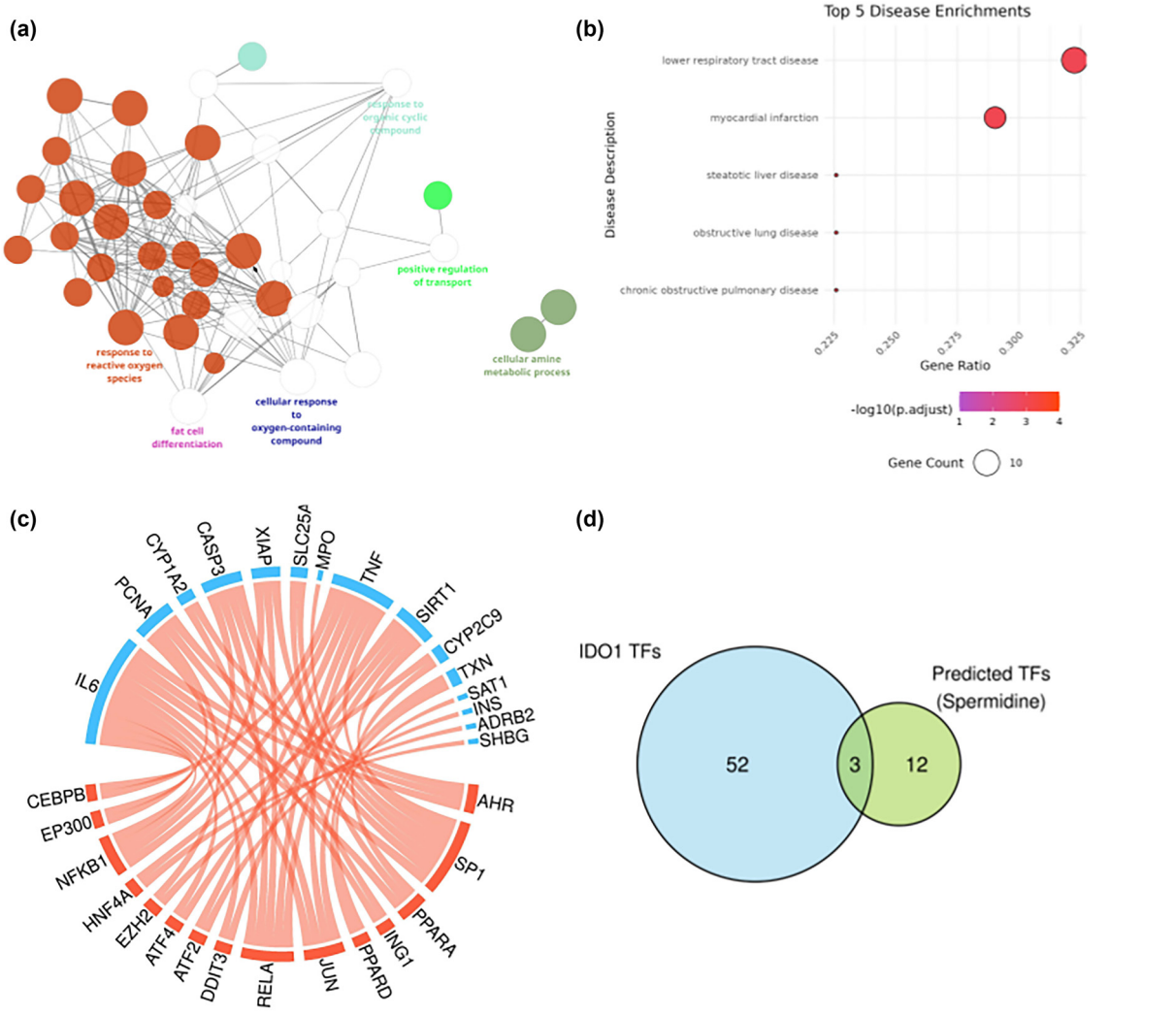


Figure 4: Functional analysis of spermidine target genes. (a) Functional annotation of spermidine target genes using ClueGO, highlighting enriched Gene Ontology Biological Processes (GO BP). (b) Top 5 disease enrichment terms identified using the DOSE R package, with an adjusted *p*-value threshold of 0.01, presented with gene ratio and significance (−log10 adjusted *p*-value). (c) Predicted TFs for spermidine target genes using EnrichR with the TRRUST Transcription Factors 2019 database, using an adjusted *p*-value threshold of 0.05. (d) Comparison of TFs, showing overlap between spermidine-predicted TFs and upstream TFs of IDO1 identified in the hTFtarget database.

effects and IDO1 activity, predicted TFs for spermidine were compared with the upstream regulators of IDO1. This analysis identified shared TFs between the two datasets, namely CEBPB, EP300, and JUN (Figure 4d). These findings highlight a potential regulatory intersection between spermidine's effects and IDO1-mediated pathways. The targeted genes, associated diseases, and TFs are available in Supplementary File 1.

4 Discussion

There is an ongoing debate about the potential positive or harmful effects of polyamines, and the health-related effects seem to depend greatly on the context. Polyamines are undoubtedly essential for numerous physiological processes, such as translation, differentiation, proliferation, lipid metabolism, and immune regulation. However, due to their antioxidant and anti-inflammatory effects, pharmacological applications are also discussed.

In recent years, spermidine in particular has emerged as a strong candidate for anti-ageing applications. Since the polyamine content naturally decreases with age, it is assumed that maintaining spermidine levels contributes to longevity [41]. Studies have shown that spermidine can extend the lifespan of various cell types, including PBMCs, and organisms such as *C. elegans* and *D. melanogaster*. In mice, spermidine feeding reduced age-related oxidative damage, further supporting its potential as an anti-ageing compound [42].

The prospective community-based study by Kiechl et al. [43] reports that higher spermidine intake is linked to lower mortality, thus providing support for the concept that nutrition rich in spermidine is cardioprotective and linked to increased survival in humans. However, safety concerns cannot be completely excluded, e.g. for excessive polyamine supplementation in the context of tumourigenesis [44,45]. The results from Kiechl et al. and others mostly refer to naturally occurring spermidine in quantities typical for a Western or Mediterranean diet and allow no conclusions on high-dose spermidine supplements or extreme diets [45,46].

One of the central mechanisms involved in ageing is chronic inflammation, known as "inflammaging" [47]. This persistent low-grade inflammation contributes significantly to the development of age-related diseases such as cardiovascular conditions, neurodegenerative disorders, and metabolic diseases. Notably, the activation of IDO-1 and GTP-CH-I activity has been reported in a variety of disorders that are accompanied by chronic immune activation [29], e.g. cardiovascular [48], autoimmune [49], and malignant diseases [50]. Moreover, Trp breakdown increases with ageing [50] and both pathways are also activated in frailty, an age-related multidimensional syndrome leading to functional decline and increased vulnerability to stressors [51]. Spermidine promotes autophagy, which is widely considered to be essential for healthy cellular and organismal ageing [52]. Of note, Kyn is a negative regulator of autophagy and promotes senescence [53].

This study investigated the effects of spermidine on the IDO-1 activity in PBMCs, both in their basal state and following stimulation of IFN-γ secretion by T cells using mitogen PHA. IDO-1 activity was dose-dependently suppressed in stimulated cells. Unstimulated cells were not affected. The effect was significant, already at a sublethal concentration of 2.5 µmol/L of spermidine supplementation, and was accompanied by a slight but significant decrease of cell viability at higher concentrations. The spermidine-mediated decrease of viability was present in both stimulated and unstimulated cells. PBMCs are more sensitive towards spermidine treatment than, e.g. human cervical cell lines. For HeLa cells, an IC50 of 121.3 µmol/L was reported for a 24 h treatment with spermidine [54].

Of note, neopterin formation was not affected under both conditions, though both pathways share common upstream regulation, most importantly IFN-γ signalling. IFN-γ secretion was sufficiently stimulated, as shown by the increased neopterin concentrations in the control cells (Figure 2). Thus, an indirect effect of spermidine mediated through the attenuation of inflammation-induced oxidative stress or IFN-γ signalling is unlikely. Notably, spermidine has been reported to act as a positive modulator of IDO-1 enzyme expression in mouse conventional dendritic cells by binding to Src kinase and promoting IDO-1 phosphorylation [55], although it remains unknown whether this mechanism also applies to human PBMCs. Any potential effect of spermidine on enzyme expression should be evaluated in future experiments, as spermidine is known to interfere with epigenetic regulation and chromatin remodeling [56,57]. Furthermore, other modes of regulation should be considered, including direct enzymatic inhibition of IDO-1 or post-translational modifications [58,59].

As a basis for further investigations, a functional analysis of spermidine target genes was performed using Gene Ontology Biological Processes (GO BP), revealing significant enrichment in several pathways related to ROS metabolism and cellular responses to oxidative stress. This aligns with spermidine's established role in regulating oxidative stress and inflammation by modulating ROS levels, supporting antioxidant defenses, and influencing key pathways in

oxidative stress-related processes [24,48,60]. This bioinformatic analysis provided insight into potential cellular processes regulated by spermidine, beyond those measured experimentally. In addition, the disease enrichment analysis showed that the spermidine-target genes are significantly associated with conditions such as respiratory and cardiovascular diseases, suggesting that spermidine's effects go beyond ageing and inflammation. TF analysis identified key regulators, including CEBPB, EP300, and JUN, which are involved in immune regulation, transcriptional activation, and IDO-1 modulation [61–63]. The identification of CEBPB, JUN, and EP300 as potential IDO1 regulators is supported by their roles in immune-related transcription and epigenetic control [64–67]. Furthermore, established IDO-1 regulators such as NFκB, AhR, and RELA were also identified, highlighting a complex network through which spermidine may influence IDO-1 activity and its broader immunomodulatory effects [58,62,64].

5 Conclusion

This study investigated the effect of spermidine on human PBMC from healthy blood donors. In mitogen-stimulated PBMC, IDO-1 activity is dose-dependently suppressed at sublethal concentrations, while no effect on Trp breakdown was observed in unstimulated cells. Neopterin formation was not affected under all conditions. Current results show that in the cell model, spermidine mediates a suppressive effect on Trp breakdown, but whether the underlying proinflammatory signaling processes are decreased, too, needs to be clarified. Although the first interpretation of results would add to the discussion of health-promoting antioxidant effects of spermidine, further investigations are necessary to understand the molecular basis of the observed effect. Conversely, excessive attenuation of IDO-1 activity may carry the risk of promoting tumourigenesis through immune surveillance impairment and tumour-promoting inflammation shifts.

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Conflict of interest: Johanna M. Gostner is the Editor-in-Chief of Pteridines. Dietmar Fuchs is a former Editor-in-Chief of Pteridines. The authors state no other conflict of interest.

Data availability statement: The datasets generated during and/or analysed during the current study are available from the corresponding author upon reasonable request.

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