Effects of free amino acids on cytokine secretion and proliferative activity of feline T cells in an in vitro study using the cell line MYA-1

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Abstract In vitro studies might be an interesting screening method for targeted in vivo studies in the field of immunonutrition and help to reduce and refine animal studies. As the role of amino acids for immune function of cats has not been evaluated in detail so far, the present study aimed at investigating the effects of eight different amino acids (arginine, leucine, isoleucine, valine, glutamine, lysine, threonine and tryptophan) in six concentrations each (0, 0.25, 0.5, 1, 2 and 8x the cat blood level) on cytokine secretion and proliferative activity of feline T cells (MYA-1) in vitro. The results demonstrated that high doses of arginine increased IL-4, IL-10 and TNF- α secretion of T cells, while increasing concentrations of lysine increased IL-10 secretion and proliferative activity of the T cells. High doses of leucine enhanced GM-CSF and IL-10 secretion, while concentrations of threonine in the cell culture media greater than blood

concentration also increased GM-CSF and additionally TNF- α secretion of the cells. The effects of glutamine and isoleucine on T cell function were only small. In conclusion, the present in vitro study could evaluate the immunomodulating potential of specific amino acids for feline T cell function. High doses of arginine, lysine, leucine and threonine had a significant impact on cytokine secretion and proliferative activity of the T cells. Targeted in vivo studies should investigate the clinical relevance of dietary supplementation of those amino acids in healthy and diseased cats as a next step.

Keywords Feline T cells · Amino acids · Cytokines · Proliferative activity · In vitro

Introduction

Nutritional studies on the feline immune system remain scarce. Since in vivo measurement of immunological parameters implies considerable time and restraints in animal welfare, alternatives to animal trials are required and have also become an important objective in the European Legislation (“reduction, replacement and refinement”). Investigations with feline cell cultures may be particularly interesting as a screening method for immunological effects of dietary factors prior to the conduction of selected in vivo studies. This allows us to precisely adopt targeted measures and to reduce animal trials.

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Current research with feline cell lines has focused on infection trials, especially with viruses (Boenzli et al. 2011; Robert-Tissot et al. 2011; Alazawy et al. 2011), or investigations with tumor cells (Shimada et al. 2006; Martin et al. 2011; Mochizuki et al. 2011). However, cell cultures can also be used to focus on physiological immune responses, for example to assess the immunomodulatory effects of nutritional factors.

Several dietetic factors are considered to have immunomodulating effects in humans and animals, for instance antioxidants like vitamin E, vitamin C or carotenoids (Chew 1993, 1995; Mayne 1996; Webb and Villamor 2007), n-3 fatty acids (Satyaraj 2011), trace elements like copper (Percival 1998; Bonham et al. 2002; Klotz et al. 2003; Wintergerst et al. 2007) and zinc (Ibs and Rink 2003; Fraker and King 2004; Wintergerst et al. 2006), phytogetic compounds (Ramesh et al. 2002) or pre- and probiotics (De Vrese and Schrezenmeir 2008). In addition, recent data indicate that amino acids have a crucial role in immune responses. The modes of action include an activation of immune cells, an effect on lymphocyte proliferation, gene expression and the redox state in cells, and a regulation of the secretion of antibodies and bioactive substances such as cytokines (Li et al. 2007). Up to now, the relevance of amino acids on immune cells in cats has not been identified. The situation in cats could be unique, having a particular requirement for amino acids such as taurine, arginine and methionine.

The aim of the present in vitro study was to evaluate dose-dependent effects of the amino acids arginine, leucine, isoleucine, valine, glutamine, lysine, threonine and tryptophan on proliferative activity and cytokine secretion of feline T cells, using the well characterized commercial cell line MYA-1 (ATCC[®] CRL-2417TM). T cells were chosen as important effectors of the adaptive immune system. Their functional activity can be detected both, by the production of bioactive substances (e.g. cytokines), and the proliferation rate (Sikalidis 2015). Importantly, it has been assumed that amino acid availability can potentially affect T cell function (Li et al. 2007; Sikalidis 2015), however, data in cats are missing.

With the exception of glutamine, all test amino acids of the present study are essential for cats (NRC 2006), and dose-dependent effects on immune function are of physiological relevance. In addition,

glutamine has been demonstrated to be an important immunomodulator (Roth 2008; Sikalidis 2015) and its effect on T cell function was also evaluated in the present study. It was hypothesized that specific amino acids can modulate the immune response of feline immune cells either by shifts in cytokine production or proliferation rate.

Materials and methods

For the present investigation, the feline cell line MYA-1 (ATCC[®] CRL-2417TM; LGC Standards GmbH, Wesel, Germany) was used. According to the provider, this cell line are T lymphoblasts isolated from peripheral blood of a 5 months old cat. These T lymphoblasts express the cluster of differentiation (CD) molecules feline CD3 and feline CD4 and are negative for feline CD8.

The cells were cultured in a complete growth medium (RPMI 1640 medium with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, 1.0 mM sodium pyruvate; LGC Standards GmbH, Wesel, Germany; supplemented with 0.05 mM 2-mercaptoethanol (Sigma-Aldrich Chemie GmbH, Munich, Germany), 100 units/mL recombinant human IL-2 (Sigma-Aldrich Chemie GmbH, Munich, Germany), 10 % fetal bovine serum (Biochrom GmbH, Berlin, Germany) and 1 % penicillin/streptomycin (Biochrom GmbH, Berlin, Germany).

In order to evaluate dose-dependent effects of different amino acids on cytokine secretion and proliferative activity of the cells, a basic cell culture medium without amino acids was used (Genaxxon BioScience GmbH, Ulm, Germany). The basic cell culture medium had the same composition as the complete growth medium, but only without amino acids (Table 1).

With the exception of one test amino acid, all other amino acids were added to the cell culture medium in the typical concentration of free amino acids present in cat blood (Heinze et al. 2009). We decided to use blood concentrations of amino acids as the effects of blood lymphocytes were evaluated and physiological conditions should be simulated best possible. Since the effects of eight test amino acids (arginine, glutamine, isoleucine, leucine, threonine, tryptophan, valine and lysine) and six doses of each amino acid (0 x blood

Table 1 Composition of the basic cell culture medium (Genaxxon BioScience GmbH, Ulm, Germany)

Component	In mg/L
CaNO ₃ × 4H ₂ O	100.00
KCl	400.00
MgSO ₄ (free of water)	48.84
NaCl	6000.00
Na ₂ HPO ₄ (free of water)	800.49
NaHCO ₃	1500.00
D(+)-Glucose (anhydrous)	4500.00
Glutathione (red.)	1.00
HEPES (10 mmol/L)	2383.10
Phenol red	5.00
Sodium pyruvate (1 mmol/L)	110.05
p-Aminobenzoic acid	1.00
D(+)-Biotin	0.20
D-Ca-pantothenate	0.25
Choline chloride	3.00
Folic acid	1.00
myo-Inositol	35.00
Nicotinamide	1.00
Pyridoxal × HCl	1.00
Riboflavin	0.20
Thiamine × HCl	1.00
Vitamin B ₁₂	0.005

Except for the respective test amino acid, all other amino acids were added to this basic cell culture medium as present in cat blood (Heinze et al. 2009)

concentration, 0.5 x blood concentration, 0.25 x blood concentration, 1 x blood concentration, 2 x blood concentration, 8 x blood concentration) were evaluated, 48 different cell culture media were prepared in total. This means that each cell culture medium contained all amino acids, but only the concentration of the respective test amino acid differed when compared to blood concentration (Table 2). Cells were cultivated in these cell culture media for 2 days prior to the following measurements of cytokine secretion and for 3 days prior to the measurement of proliferative activity. Different cell culture batches were used for the cytokine measurements and measurements of the proliferative activity of the T cells as well as for the replicates. Cell density was $1.2 \times 10^7/2$ mL/treatment for cytokine measurements and $4 \times 10^5/200$ µL/treatment for the measurement of the proliferative activity of the T cells.

Cytokine measurement

T cell secretion of the cytokines interleukin-4 (IL-4), interleukin-10 (IL-10), tumor necrosis factor alpha (TNF-α), granulocyte macrophage colony-stimulating factor (GM-CSF), interferon gamma (IFN-γ), interleukin-6 (IL-6) and interleukin-1beta/interleukin-1F2 (IL-1β/IL-1F2) was measured using Enzyme Linked Immunosorbent Assays (all R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany). The tests were performed according to the instructions of the manufacturer. For each assay, 2 wells per test amino acid concentration were considered on an ELISA plate, and cytokine secretion was measured in duplicate. Three replicates were considered for each assay, resulting in a sample size of $n = 12$ /test amino acid concentration for each parameter.

Proliferative activity

For the measurement of the proliferative activity of the T cells, a commercial MTT test (Vybrant® MTT Cell Proliferation Assay Kit, Invitrogen, Eugene, OR, USA) was used. The test was performed according to the manufacturer's instructions with slight modification. When the cells were labeled with MTT, all but 100 µL of medium was removed from each well, and 200 µL DMSO (Carl Roth GmbH + Co. KG, Karlsruhe, Germany) were added to each well, subsequently.

For each test amino acid concentration, 6 wells on a multiwell plate (Brand plates®, Brand, Wertheim, Germany) were measured. The proliferative activity of the feline T cells was measured as optical density at a wavelength of 540 nm (OD540) (Infinite M200 Pro, Tecan, Männedorf, Switzerland; software: i-control 1.7, Tecan, Männedorf, Switzerland). 3 replicates were considered for the MTT assay, resulting in a sample size of $n = 18$ /test amino acid concentration.

Statistics

For statistical data analysis, SPSS 20 (SPSS, Inc., Chicago, IL, USA) was used. Data were compared by one-factor analysis of variance (fixed factor: concentration of the test amino acid) and Scheffé (variance equality) or Tamhane 2 (variance inequality) post hoc tests. As different cell culture batches were used for the replicates, data were treated as independent measures. Polynomial contrasts were calculated in order to identify linear or quadratic

Table 2 Concentrations of the test amino acids in the cell media

	Dose amino acids (mg/L)					
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration
Arginine	0	4.13	8.25	16.50	33.00	132.00
Glutamine	0	24.25	48.50	97.00	194.00	776.00
Isoleucine	0	2.07	4.13	8.26	16.52	66.08
Leucine	0	4.80	9.60	19.20	38.40	153.60
Threonine	0	5.15	10.30	20.60	41.20	164.80
Tryptophan	0	3.08	6.15	12.30	24.60	98.40
Valine	0	4.80	9.60	19.20	38.40	153.60
Lysine	0	4.93	9.85	19.70	39.40	157.60

Only the dose of one test amino acid varied in each cell culture medium, while the other amino acids were added to the medium at blood concentration (Heinze et al. 2009). Therefore, 48 different cell media were prepared for the present study

effects of the test amino acid concentration in the cell culture medium on the test parameters. Data are presented in tables as mean $\pm$ standard deviation. The level of significance was $\alpha = 0.05$.

Results

Dose-dependent effects of specific amino acids on the cytokine secretion of feline T cells

The dose-dependent effects of arginine, glutamine, isoleucine, leucine, threonine, tryptophan, valine and lysine on the secretion of IL-4, IL-10, TNF- α , GM-CSF and IFN- γ of the feline T cells are presented in Tables 3, 4, 5, 6 and 7. T cell secretion of interleukin-6 (IL-6) and IL-1 β /IL-1F2, depending on varying concentrations of the test amino acids in the cell culture media, was low (all values were below the detection limit of 31.25 pg/mL), therefore no statistical data analysis was possible.

IL-4 secretion

IL-4 secretion was highest when threonine was added to the cell culture medium in a concentration that can be found in cat blood, while a reduced IL-4 secretion was observed when threonine concentration in the cell culture medium was lower or higher (quadratic effect: $P < 0.001$) (Table 3).

Contrasting effects were observed for lysine, where the lowest IL-4 secretion was measured when lysine

was added to the cell culture medium at a concentration present in cat blood or at half of the concentration in cat blood (quadratic effect: $P = 0.015$).

The cell culture medium without arginine led to a lower IL-4 secretion of the T cells when compared to arginine concentrations in the cell culture media which were twofold or eightfold higher than in cat blood ($P < 0.05$).

Only small group differences were observed for the IL-4 secretion of the T cells when glutamine, leucine and valine were added to the cell culture media at varying concentrations ($P < 0.05$).

IL-10 secretion

A linear effect of isoleucine ($P = 0.039$) and leucine ($P = 0.008$) concentrations in the cell culture media was detected for IL-10 secretion of the T cells (Table 4). Highest IL-10 secretion was observed when isoleucine and leucine were added to the media in concentrations which were twice as high as in cat blood, while lower IL-10 secretion was observed when isoleucine and leucine were present in the cell culture media at concentrations found in cat blood or below.

A linear effect on IL-10 secretion of the T cells was also observed for lysine concentrations in the cell culture media, where an increase was detected when lysine concentration was 0 mg/L up to the eightfold blood concentration ($P = 0.015$).

With regard to arginine concentrations in the cell culture media, lowest IL-10 secretion was measured

Table 3 IL-4 secretion (pg/mL) of feline T cells, depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)						Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Lin. contrast	Quadr. contrast
Arginine	1090.38 ± 72.81 ^a	1126.30 ± 419.14 ^{ab}	981.04 ± 489.55 ^{ab}	1171.61 ± 428.34 ^{ab}	1311.33 ± 118.84 ^b	1282.13 ± 28.25 ^b	0.089	0.443
Glutamine	1015.89 ± 105.80 ^a	1289.25 ± 238.43 ^{ab}	1047.10 ± 534.84 ^{ab}	1248.69 ± 129.51 ^b	1156.95 ± 131.44 ^{ab}	1106.17 ± 94.26 ^{ab}	0.755	0.252
Isoleucine	1202.11 ± 216.13	1260.94 ± 240.40	1197.40 ± 168.77	1257.61 ± 153.45	1256.65 ± 183.52	1247.95 ± 162.91	0.625	0.888
Leucine	1103.05 ± 46.44 ^a	1312.65 ± 109.34 ^b	1206.20 ± 123.16 ^{ab}	1248.23 ± 118.57 ^{ab}	1286.96 ± 126.39 ^{ab}	1123.19 ± 565.41 ^{ab}	0.933	0.142
Threonine	1040.92 ± 34.75 ^a	1175.67 ± 132.57 ^{ab}	1084.46 ± 20.91 ^a	1326.68 ± 69.35 ^b	1263.90 ± 48.83 ^b	1118.88 ± 50.45 ^a	<0.001	<0.001
Tryptophan	1192.69 ± 96.99	1345.63 ± 138.18	1176.29 ± 39.20	1208.33 ± 52.96	1204.38 ± 17.70	1172.99 ± 34.94	0.034	0.295
Valine	1120.83 ± 64.56 ^b	1236.83 ± 145.57 ^{ab}	1210.48 ± 56.56 ^a	1305.25 ± 41.05 ^b	1072.58 ± 542.61 ^{ab}	1004.51 ± 496.46 ^{ab}	0.300	0.101
Lysine	1265.06 ± 124.54	1358.45 ± 162.55	837.03 ± 569.89	849.22 ± 578.24	1236.90 ± 71.77	1199.81 ± 31.47	0.557	0.015

Means ± standard deviations

Different letters in the same row as well as bold values for polynomial contrasts indicate significant group differences ($P < 0.05$)

when arginine was added to the medium at a concentration which can be found at cat blood and in half blood concentration, while highest IL-10 secretion was measured when arginine concentration in the cell culture medium was twofold and eightfold higher than in cat blood (linear and quadratic contrasts: $P = 0.004$).

Only small group differences were observed for the IL-10 secretion of the T cells when glutamine and threonine were added to the cell culture media at varying concentrations ($P < 0.05$).

IL-10 secretion of the T cells was below the detection limit (125 pg/mL) for tryptophan (0 mg/L), valine (0 mg/L; blood concentration; 8 x blood concentration) and lysine (blood concentration), therefore post hoc tests were not performed.

TNF- α secretion

An increase of TNF- α secretion was observed from 0 mg arginine/L up to an arginine concentration in the cell culture medium which was twice as high as in cat blood (linear contrast: $P = 0.048$) (Table 5).

A quadratic effect of leucine concentrations in the cell culture media on TNF- α secretion of the T cells was detected. Highest TNF- α secretion was measured when leucine concentration was similar to blood concentration, while a reduced TNF- α secretion was observed when leucine concentrations in the media were higher or lower ($P < 0.001$).

Lowest TNF- α secretion was measured when threonine was added to the medium in a concentration which was half of the concentration found in cat blood, while higher TNF- α secretion was detected when threonine concentrations in the media were below or above this concentration (quadratic effect: $P = 0.015$).

Contrary results were observed for valine, where TNF- α secretion of the T cells was highest when valine concentration was half of cat blood concentration, while a reduced TNF- α secretion was observed when valine concentrations in the media were higher or lower (quadratic effect: $P = 0.038$).

GM-CSF secretion

A quadratic effect was observed for GM-CSF secretion of the T cells when glutamine was added to the cell culture media at varying concentrations, where

Table 4 IL-10 secretion (pg/mL) of feline T cells, depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)					Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Quadr. contrast
Arginine	137.75 ± 9.63 ^{abc}	144.48 ± 10.64 ^{abc}	129.77 ± 2.24 ^a	132.08 ± 4.93 ^{ac}	145.49 ± 6.86 ^b	167.84 ± 29.62 ^{bc}	0.004
Glutamine	133.88 ± 2.59 ^a	150.82 ± 5.47 ^b	142.89 ± 8.89 ^{ab}	137.24 ± 7.54 ^{ab}	144.26 ± 10.13 ^{ab}	140.97 ± 6.67 ^{ab}	0.151
Isoleucine	140.11 ± 4.88 ^a	142.94 ± 5.00 ^{ab}	137.52 ± 10.33 ^{ab}	144.55 ± 17.34 ^{ab}	149.47 ± 2.80 ^b	144.76 ± 4.86 ^{ab}	0.039
Leucine	130.03 ± 3.44 ^{ab}	146.60 ± 6.56 ^{ab}	136.28 ± 8.09 ^a	138.59 ± 8.64 ^{ab}	159.77 ± 19.88 ^b	150.45 ± 12.88 ^{ab}	0.946
Threonine	143.68 ± 6.71 ^{ab}	149.98 ± 4.41 ^a	133.17 ± 7.14 ^b	134.16 ± 2.75 ^{ab}	138.86 ± 8.40 ^{ab}	136.69 ± 7.14 ^{ab}	0.143
Tryptophan	*	130.24 ¹	136.27 ± 7.45	133.93 ± 1.95	139.67 ± 7.99	135.01 ± 7.76	0.450
Valine	*	128.91 ¹	139.06 ¹	*	156.64 ± 21.14	*	0.325
Lysine	135.30 ± 4.37	152.95 ± 12.02	147.85 ± 14.06	*	148.65 ± 10.45	157.96 ± 15.75	0.015

Means ± standard deviations

Different letters in the same row as well as bold values for polynomial contrasts indicate significant group differences (*P* < 0.05)

* All values below detection limit of 125 pg/mL

¹ Only 1 value > detection limit

Table 5 TNF- α secretion (pg/mL) of feline T cells, depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)					Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Lin. contrast Quadr. contrast
Arginine	18.44 $\pm$ 1.75	18.82 $\pm$ 2.29	17.85 $\pm$ 1.00	22.12 $\pm$ 8.06	25.53 $\pm$ 6.81	20.14 $\pm$ 2.60	0.048 0.518
Glutamine	30.00 $\pm$ 0.65	27.38 $\pm$ 9.48	22.87 $\pm$ 5.94	25.07 $\pm$ 8.18	26.60 $\pm$ 5.99	22.35 $\pm$ 5.58	0.130 0.577
Isoleucine	29.00 $\pm$ 2.94	28.00 $\pm$ 1.64	29.87 $\pm$ 2.22	31.40 $\pm$ 3.01	29.02 $\pm$ 8.01	24.61 $\pm$ 5.89	0.371 0.126
Leucine	19.71 $\pm$ 1.84 ^{ab}	20.78 $\pm$ 3.51 ^{ab}	27.39 $\pm$ 6.24 ^{ab}	29.53 $\pm$ 1.07 ^a	20.73 $\pm$ 1.82 ^{ab}	19.17 $\pm$ 2.99 ^b	0.967 < 0.001
Threonine	22.45 $\pm$ 4.68	24.98 $\pm$ 2.97	17.38 $\pm$ 1.57	20.86 $\pm$ 4.77	25.09 $\pm$ 3.58	28.27 $\pm$ 1.87	0.059 0.015
Tryptophan	25.45 $\pm$ 2.83	20.11 $\pm$ 5.39	25.92 $\pm$ 6.29	26.04 $\pm$ 1.08	24.97 $\pm$ 5.41	22.85 $\pm$ 5.00	0.926 0.591
Valine	23.75 $\pm$ 5.32 ^{ab}	24.98 $\pm$ 6.35 ^{ab}	29.36 $\pm$ 3.39 ^a	21.85 $\pm$ 5.42 ^{ab}	19.58 $\pm$ 4.12 ^b	18.22 $\pm$ 1.31 ^{ab}	0.004 0.038
Lysine	16.57 $\pm$ 0.48	18.07 $\pm$ 2.60	18.06 $\pm$ 2.02	19.83 $\pm$ 2.47	16.69 $\pm$ 0.50	17.53 $\pm$ 2.36	0.832 0.189

Means $\pm$ standard deviationsDifferent letters in the same row as well as bold values for polynomial contrasts indicate significant group differences ($P < 0.05$)

highest GM-CSF secretion was measured when the same glutamine concentration was achieved as in cat blood ($P = 0.015$) (Table 6). Comparable results were detected for arginine concentrations in the cell culture media (quadratic effect: $P = 0.017$), lysine (quadratic effect: $P = 0.026$) and tryptophan (quadratic contrast: $P < 0.001$). In contrast, GM-CSF secretion was lowest when threonine was added to the cell culture medium in a concentration that can be found in cat blood (quadratic effect: $P = 0.004$).

Highest GM-CSF secretion of the T cells was measured when leucine concentration was eightfold higher than in cat blood (linear effect: $P = 0.033$).

Valine concentrations in the cell culture media also affected GM-CSF secretion of the T cells. The highest secretion was observed when valine concentration was a quarter of the blood concentration, while higher concentrations decreased GM-CSF secretion (linear and quadratic contrasts: $P < 0.001$).

IFN- γ secretion

IFN- γ secretion of the T cells was low when arginine, glutamine, isoleucine, leucine and lysine were added to the cell culture media in varying concentrations (Table 7). The measured values were often below the detection limit (62.5 pg/mL), therefore post hoc tests were not performed.

Lowest IFN- γ secretion of the T cells was observed when tryptophan was added to the medium at the concentration which can be found in cat blood, while an enhanced IFN- γ secretion was measured when tryptophan was added to the media below or above blood concentration (quadratic effect: $P = 0.024$). Contrary results were detected for lysine, where highest IFN- γ secretion of T cells was observed when lysine was added to the cell culture medium at the same concentration as lysine is present in cat blood or in half cat blood concentration (quadratic effect: $P = 0.022$).

IFN- γ secretion of T cells was highest when valine was added to the cell culture media at concentrations below cat blood concentration (linear contrast: $P < 0.001$; quadratic contrast: $P = 0.034$).

Dose-dependent effects of specific amino acids on the proliferative activity of feline T cells

When arginine or tryptophan were added to the media, the highest proliferative activity of the T cells was

Table 6 GM-CSF secretion (pg/mL) of feline T cells, depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)					Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Quadr. contrast
Arginine	37.61 ¹	60.46 ± 1.80	47.36 ± 9.34	50.81 ± 4.67	44.52 ± 3.59	42.96 ± 5.66	0.017
Glutamine	40.66 ± 5.44	48.23 ²	40.66 ± 5.29	57.96 ± 5.21	49.56 ± 9.55	34.41 ¹	0.015
Isoleucine	*	39.05 ± 7.04	34.39 ± 2.78	32.79 ²	36.79 ²	*	0.211
Leucine	42.94 ²	61.01 ± 6.53	*	36.02 ²	68.51 ± 14.35	75.95 ± 23.72	0.354
Threonine	116.75 ± 2.31	110.23 ± 32.72	77.73 ± 33.44	76.62 ± 17.61	92.71 ± 9.10	109.60 ± 6.02	0.004
Tryptophan	84.27 ± 6.52 ^c	85.81 ± 2.48 ^c	108.36 ± 4.19 ^b	125.93 ± 7.54 ^a	106.67 ± 1.99 ^b	98.61 ± 9.60 ^{bc}	<0.001
Valine	87.19 ± 5.57 ^{bc}	108.75 ± 6.20 ^c	96.50 ± 4.47 ^{bc}	82.73 ± 8.81 ^b	53.21 ± 12.39 ^a	50.42 ± 12.96 ^a	<0.001
Lysine	37.13 ± 4.03	46.65 ± 16.61	50.08 ± 13.12	67.61 ± 6.57	52.50 ± 5.68	49.94 ± 14.74	0.026

Means ± standard deviations

Different letters in the same row as well as bold values for polynomial contrasts indicate significant group differences (*P* < 0.05)

* All values below detection limit of 31.25 pg/mL

¹ Only 1 value > detection limit² Only 2 values > detection limit

Table 7 IFN- γ secretion (pg/mL) of feline T cells, depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)					Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Quadr. contrast
Arginine	82.29 ²	55.53 ¹	46.61 ¹	60.53 $\pm$ 12.71	25.59 ¹	*	0.053
Glutamine	*	66.23 $\pm$ 17.03	*	*	*	*	**
Isoleucine	101.26 $\pm$ 21.90	67.18 ²	*	51.75 ²	142.78 $\pm$ 37.47	72.61 $\pm$ 34.82	0.620
Leucine	54.36 $\pm$ 16.76	172.09 $\pm$ 34.51	149.89 $\pm$ 39.76	68.58 ¹	107.68 $\pm$ 54.23	157.23 $\pm$ 42.83	0.700
Threonine	112.11 $\pm$ 59.08	100.99 $\pm$ 28.23	73.80 $\pm$ 43.74	133.73 $\pm$ 34.63	178.87 $\pm$ 20.10	116.15 $\pm$ 29.32	0.864
Tryptophan	190.76 $\pm$ 40.14 ^{ab}	223.17 $\pm$ 58.98 ^{ab}	191.41 $\pm$ 19.88 ^{ab}	136.70 $\pm$ 26.63 ^a	206.82 $\pm$ 17.87 ^{ab}	241.78 $\pm$ 42.12 ^b	0.024
Valine	216.88 $\pm$ 29.41 ^b	217.42 $\pm$ 19.35 ^b	212.03 $\pm$ 25.03 ^b	194.78 $\pm$ 71.80 ^{ab}	145.23 $\pm$ 42.00 ^{ab}	93.43 $\pm$ 32.27 ^a	0.034
Lysine	*	55.66 $\pm$ 24.46	106.26 $\pm$ 31.69	105.15 ²	35.79 $\pm$ 13.51	48.73 $\pm$ 25.08	0.022

Means $\pm$ standard deviationsDifferent letters in the same row as well as bold values for polynomial contrasts indicate significant group differences ($P < 0.05$)

* All values below detection limit of 62.5 pg/mL

** No calculation of polynomial contrasts possible

¹ Only 1 value > detection limit² Only 2 values > detection limit

Table 8 Proliferative activity (measured as optical density OD540) of feline T cells depending on the dose of specific amino acids in the cell culture media

	Dose of amino acids (mg/L)						Dose (<i>P</i> value)	
	Without (0)	0.25 x blood concentration	0.5 x blood concentration	1 x blood concentration	2 x blood concentration	8 x blood concentration	Lin. contrast	Quadr. contrast
Arginine	0.80 ± 0.09 ^a	0.89 ± 0.05 ^b	0.93 ± 0.06 ^{bc}	0.95 ± 0.06 ^c	0.92 ± 0.09 ^{bc}	0.90 ± 0.11 ^{abc}	<0.001	<0.001
Glutamine	0.82 ± 0.07 ^a	0.90 ± 0.13 ^{ab}	0.86 ± 0.13 ^{ab}	0.91 ± 0.09 ^{ab}	0.93 ± 0.10 ^{ab}	0.95 ± 0.11 ^b	<0.001	0.643
Isoleucine	0.94 ± 0.15	1.02 ± 0.15	0.93 ± 0.12	0.94 ± 0.10	0.90 ± 0.05	0.91 ± 0.07	0.021	0.558
Leucine	0.86 ± 0.12	0.87 ± 0.12	0.88 ± 0.10	0.88 ± 0.09	0.86 ± 0.09	0.88 ± 0.17	0.619	0.878
Threonine	0.87 ± 0.12	0.89 ± 0.11	0.87 ± 0.11	0.90 ± 0.10	0.91 ± 0.14	0.90 ± 0.17	0.355	0.808
Tryptophan	0.89 ± 0.10	0.89 ± 0.09	0.90 ± 0.11	0.96 ± 0.13	0.87 ± 0.08	0.86 ± 0.12	0.468	0.035
Valine	0.84 ± 0.11	0.91 ± 0.11	0.85 ± 0.13	0.90 ± 0.10	0.89 ± 0.12	0.90 ± 0.12	0.218	0.638
Lysine	0.90 ± 0.08	0.93 ± 0.16	0.88 ± 0.12	0.95 ± 0.11	1.01 ± 0.11	0.99 ± 0.12	0.001	0.523

Means ± standard deviations

Different letters in the same row as well as bold values for polynomial contrasts indicate significant group differences ($P < 0.05$)

measured when the arginine or tryptophan concentration was equivalent to blood concentration (quadratic effect: $P < 0.001$ and $P = 0.035$ for arginine and tryptophan, respectively) (Table 8). Linear effects were observed when glutamine ($P < 0.001$), isoleucine ($P = 0.021$) and lysine ($P = 0.001$) were added to the cell culture media. The proliferative activity of the T cells was the highest when glutamine or lysine concentrations in the cell culture media increased or isoleucine concentrations decreased.

Discussion

The present study was conducted in order to evaluate the effects of specific amino acids on feline T cells in vitro. To date, cell lines are predominantly used for infection trials and oncological research (Shimada et al. 2006; Alazawy et al. 2011; Boenzli et al. 2011; Martin et al. 2011; Mochizuki et al. 2011; Robert-Tissot et al. 2011). However, in vitro studies might also be interesting as screening method for immunonutritional effects in cats. In this study, the effects of eight different amino acids with six doses each were evaluated concerning stimulating or inhibiting effects on cytokine secretion and proliferative activity of feline T cells. The realization of a similar in vivo study would require 48 different experimental diets. Moreover, immunological responses to dietetic

treatments might only be detectable when considering a sufficiently long feeding period. Thus, an in vitro study shows significant advantages to refine and reduce animal trials with regard to time and physical intervention.

Feline cell lines are available to study physiological and pathological immune responses in vitro. In the present study, the commercially available T lymphoblastoid cell line MYA-1 (ATCC® CRL-2417™) was used. As an advantage, this cell line is well characterized (CD3⁺; CD4⁺; CD8[−]), allowing the evaluation of nutritional effects on a selected immune cell population.

Several amino acids and varying concentrations were considered for the present investigation, since amino acids have been demonstrated to play a significant role in immune function (Li et al. 2007). However, studies in cats remain scarce, and the results of the present in vitro study can generate new hypotheses for targeted in vivo studies.

The effects of the amino acids arginine, leucine, isoleucine, valine, glutamine, lysine, threonine and tryptophan on feline T cells were evaluated. Among these amino acids, most immunological effects were observed for arginine and lysine. High concentrations of arginine increased IL-4, IL-10 and TNF- α secretion of T cells, while highest GM-CSF secretion and proliferative activity of the cells were observed when arginine was added to the cell culture medium in a

concentration which can be found in cat blood. Increasing concentrations of lysine increased IL-10 secretion and proliferative activity of the T cells, while highest GM-CSF and IFN- γ , but lowest IL-4 secretion was observed when lysine concentrations in the media were similar to blood concentrations.

Both IL-4 and IL-10 are mainly produced by T cells (Turner et al. 2014). While IL-4 increases proliferative activity of cytotoxic T cells and B cells, and immunoglobulin (Ig) E and IgG production, IL-10 shows anti-inflammatory effects, decreases cytokine production and inhibits function of mononuclear cells (Turner et al. 2014). The observed increase in IL-10 secretion by increasing concentrations of arginine and lysine in the cell culture media may indicate beneficial effects of these amino acids in inflammatory disorders. However, differences in IL-10 levels were only small among the selected doses of arginine and lysine. The observed increase of IL-4 secretion with increasing arginine concentrations in the cell culture media was more pronounced and may have practical relevance in pathogen defense. In addition, higher concentrations of arginine also increased TNF- α secretion of the T cells. As arginine is metabolized by nitric oxide synthases (Raber et al. 2012), an increased production of nitric acid might explain the enhanced TNF- α secretion of the feline T cells observed in the present study. Nitric acid has been demonstrated to regulate the secretion of several cytokines, including TNF (Bogdan 2001). Since TNF- α particularly stimulates phagocytic activity of macrophages (Turner et al. 2014), the observed effects of high doses of arginine may be interesting to strengthen immune function in disease. The results are supported by data of Rutherford-Markwick et al. (2013) who could demonstrate an enhanced phagocytic activity of blood leukocytes by dietary supplementation of arginine in healthy cats. In this study, 1.9 % arginine was added to a low-protein diet (22.7 % crude protein in dry matter), and fed for 5 weeks. Phagocytic activity of blood leukocytes was increased after dietary arginine supplementation when compared to feeding a low- (22.7 % crude protein) or high-protein diet (53.0 % crude protein) without additional arginine supplementation ($P < 0.05$). The results of the present study on cytokine secretion of T cells may help to explain the mechanism of the immunostimulating effect of arginine. Future studies should investigate its clinical relevance, particularly in diseased cats.

Considering the effects of lysine in the present investigation, further *in vivo* studies on its immunomodulating potential seem to be interesting. Dietary lysine supplementation has been recommended for cats infected with feline herpesvirus 1 for a long time, however, current data could not prove antiviral effects of lysine (Maggs et al. 2006; Rees and Lubinski 2008; Drazenovich et al. 2009; Bol and Bunnik 2015). Stiles et al. (2002) could demonstrate some beneficial effects of dietary lysine supplementation in cats with feline herpesvirus conjunctivitis, whereas the study of Maggs et al. (2003) could not support this observation. Nevertheless, the observed increase in IL-10 secretion and proliferative activity of T cells by increasing levels of lysine in the cell culture media of the present investigation indicate an immunomodulating potential of lysine. Further studies may not focus on antiviral effects of this amino acid, but on its potential to support immune function in healthy and diseased cats.

Besides arginine and lysine, tryptophan concentrations in the cell culture media also affected cytokine production and proliferative activity of the feline T cells. Interestingly, highest GM-CSF secretion and proliferative activity was observed when tryptophan was added to the medium in a concentration found in cat blood, indicating that dietary supplementation of tryptophan may not exert additional beneficial effects on immune function. On the other hand, IFN- γ secretion was lowest at blood concentration of tryptophan. Since IFN- γ is important for function of neutrophils and monocytes, expression of Major Histocompatibility Complex (MHC) molecules and can also have anti-viral effects (Turner et al. 2014), dietary tryptophan supplementation may be interesting in specific diseases. Based on the present results, further investigations on the effects of tryptophan are necessary to proof its clinical relevance for immune modulation in cats.

Interesting results were observed for increasing concentrations of leucine in the cell culture media, as GM-CSF and IL-10 secretion of the feline T cells was increased. GM-CSF can stimulate production of eosinophils, granulocytes and monocytes (Turner et al. 2014), which might be relevant in infectious diseases. Concentrations of threonine in the cell culture media greater than blood concentration also increased GM-CSF and additionally TNF- α secretion of the T cells.

GM-CSF and IFN- γ secretion of the feline T cells were higher when valine concentrations in the cell culture media were below blood concentration. These results are in contrast to studies in humans and rodents demonstrating that deprivation of branched-chain amino acids (BCAA) depressed and supplementation of BCAA enhanced immune function (Li et al. 2007). The reason for these contradicting results remains unclear and require further investigations.

The effects of glutamine and isoleucine on T cell function were small and did not indicate beneficial effects for dietary supplementation in vivo.

In conclusion, the present study demonstrated that in vitro evaluation of feline T cell function can be used as an interesting screening method for targeted in vivo studies in immunonutrition. Varying concentrations of specific amino acids affected cytokine secretion and proliferative activity of feline T cells. In particular, the clinical relevance of the observed immunostimulating effects of high doses of arginine, lysine, leucine and threonine should be evaluated in future studies.

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References

- Alazawy A, Arshad SS, Bejo MH, Omar AR, Tengku Ibrahim TA, Sharif S, Bande F, Awang-Isa K (2011) Ultrastructure of *Felis catus* whole fetus (Fcfw-4) cell culture following infection with feline coronavirus. *J Electron Microsc* (Tokyo) 60:275–282
- Boenzli E, Robert-Tissot C, Sabatino G, Cattori V, Meli ML, Gutte B, Rovero P, Flynn N, Hofmann-Lehmann R, Lutz H (2011) In vitro inhibition of feline leukaemia virus infection by synthetic peptides derived from the transmembrane domain. *Antivir Ther* 16:905–913
- Bogdan C (2001) Nitric oxide and the immune response. *Nat Immunol* 2:907–916
- Bol S, Bunnik EM (2015) Lysine supplementation is not effective for the prevention or treatment of feline herpesvirus 1 infection in cats: a systematic review. *BMC Vet Res* 11:284
- Bonham M, O'Connor JM, Hannigan BM, Strain JJ (2002) The immune system as a physiological indicator of marginal copper status? *Br J Nutr* 87:393–403
- Chew BP (1993) Role of carotenoids in the immune response. *J Dairy Sci* 76:2804–2811
- Chew BP (1995) Antioxidant vitamins affect food animal immunity and health. *J Nutr* 125:1804S–1808S
- De Vrese M, Schrezenmeier J (2008) Probiotics, prebiotics, and synbiotics. *Adv Biochem Eng Biotechnol* 111:1–66
- Drazenovich TL, Fascetti AJ, Westermeyer HD, Sykes JE, Bannasch MJ, Kass PH, Hurley KF, Maggs DJ (2009) Effects of dietary lysine supplementation on upper respiratory and ocular disease and detection of infectious organisms in cats within an animal shelter. *Am J Vet Res* 70:1391–1400
- Fraker PJ, King LE (2004) Reprogramming of the immune system during zinc deficiency. *Ann Rev Nutr* 24:277–298
- Heinze CR, Larsen JA, Kass PH, Fascetti AJ (2009) Plasma amino acid and whole blood taurine concentrations in cats eating commercially prepared diets. *Am J Vet Res* 70:1374–1382
- Ibs KH, Rink L (2003) Zinc-altered immune function. *J Nutr* 133:1452S–1455S
- Klotz LO, Kroencke KD, Buchczyk DP, Sies H (2003) Role of copper, zinc, selenium, and tellurium in the cellular defense against oxidative and nitrosative stress. *J Nutr* 133:1448S–1451S
- Li P, Yin Y-L, Li D, Kim SW, Wu G (2007) Amino acids and immune function. *Br J Nutr* 98:237–252
- Maggs DJ, Nasisse MP, Kass PH (2003) Efficacy of oral supplementation with L-lysine in cats latently infected with feline herpesvirus. *Am J Vet Res* 64:37–42
- Maggs DJ, Sykes JE, Clarke HE, Yoo SH, Kass PH, Lappin MR, Rogers QR, Waldron MK, Fascetti AJ (2006) Effects of dietary lysine supplementation in cats with enzootic upper respiratory disease. *J Feline Med Surg* 9:97–108
- Martin CK, Dirksen WP, Shu ST, Werbeck JL, Thudi NK, Yamaguchi M, Wolfe TD, Heller KN, Rosol TJ (2011) Characterization of bone resorption in novel in vitro and in vivo models of oral squamous cell carcinoma. *Oral Oncol* 48:491–499
- Mayne ST (1996) Beta-carotene, carotenoids and disease prevention in human. *FASEB J* 10:690–701
- Mochizuki H, Takahashi M, Nishigaki K, Ide T, Goto-Koshino Y, Watanabe S, Sato H, Sato M, Kotera Y, Fujino Y, Ohno K, Uchida K, Tsujimoto H (2011) Establishment of a novel feline leukemia virus (FeLV)-negative B-cell cell line from a cat with B-cell lymphoma. *Vet Immunol Immunopathol* 140:307–311
- National Research Council (NRC) (2006) Nutrient requirements of dogs and cats. The National Academies Press, Washington
- Percival SS (1998) Copper and immunity. *Am J Clin Nutr* 67:1064S–1085S
- Raber P, Ochoa AC, Rodríguez PC (2012) Metabolism of L-arginine by myeloid-derived suppressor cells in cancer: mechanisms of T cell suppression and therapeutic perspectives. *Immunol Invest* 41:614–634
- Ramesh HP, Yamaki K, Tsushida T (2002) Effect of fenugreek (*Trigonella foenum-graecum* L.) galactomannan fractions on phagocytosis in rat macrophages and on proliferation and IgM secretion in HB4C5 cells. *Carbohydr Polym* 50:79–83
- Rees TM, Lubinski JL (2008) Oral supplementation with L-lysine did not prevent upper respiratory infection in a shelter population of cats. *J Feline Med Surg* 10:510–513
- Robert-Tissot C, Rüegger VL, Cattori V, Meli ML, Riond B, Gomes-Keller MA, Vögtlin A, Wittig B, Juhls C, Hofmann-Lehmann R, Lutz H (2011) The innate antiviral immune system of the cat: molecular tools for the

- measurement of its state of activation. *Vet Immunol Immunopathol* 143:269–281
- Roth E (2008) Nonnutritive effects of glutamine. *J Nutr* 138:2025S–2031S
- Rutherford-Markwick KJ, Hendriks WH, Morel PC, Thomas DG (2013) The potential for enhancement of immunity in cats by dietary supplementation. *Vet Immunol Immunopathol* 152:333–340
- Satyaraj E (2011) Emerging paradigms in immunonutrition. *Top Companion Anim Med* 26:25–32
- Shimada D, Fukuda A, Kanouchi H, Matsumoto M, Oka T (2006) Vitamin B6 suppresses growth of the feline mammary tumor cell line FRM. *Biosci Biotechnol Biochem* 70:1038–1040
- Sikalidis AK (2015) Amino acids and immune response: a role for cysteine, glutamine, phenylalanine, tryptophan and arginine in T-cell function and cancer? *Pathol Oncol Res* 21:9–17
- Stiles J, Townsend WM, Rogers QR, Krohne SG (2002) Effect of oral administration of L-lysine on conjunctivitis caused by feline herpesvirus in cats. *Am J Vet Res* 63:99–103
- Turner MD, Nedjai B, Hurst T, Pennington DJ (2014) Cytokines and chemokines: at the crossroads of cell signalling and inflammatory disease. *Biochim Biophys Acta* 1843:2563–2582
- Webb AL, Villamor E (2007) Update: effects of antioxidant and non-antioxidant vitamin supplementation on immune function. *Nutr Rev* 65:181–217
- Wintergerst ES, Maggini S, Hornig DH (2006) Immune-enhancing role of Vitamin C and zinc and effect on clinical conditions. *Ann Nutr Metab* 50:85–94
- Wintergerst ES, Maggini S, Hornig DH (2007) Contribution of selected vitamins and trace elements to immune function. *Ann Nutr Metab* 51:301–323