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Serum Immune System Biomarkers Neopterin and Interleukin-10 Are Strongly Related to Tryptophan Metabolism in Healthy Young Adults[1–3]

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Abstract

Background: Changes in tryptophan metabolism through the vitamin B-6–dependent kynurenine pathway have been linked to activation of the immune system.

Objective: We hypothesized that blood concentrations of tryptophan and its catabolites were associated with biomarkers relevant to inflammatory processes in healthy noninflamed subjects.

Methods: Healthy young adults (n = 737) aged 18–28 y without any known diseases or clinical evidence of inflammation provided blood samples for analysis of serum tryptophan/kynurenine metabolites, neopterin, C-reactive protein (CRP), and plasma pyridoxal 5′-phosphate (PLP) with LC–tandem mass spectrometry methodologies. A panel of cytokines was measured in serum by using high-sensitivity ELISA assays. Anthropometric and lifestyle data were collected by questionnaire. Multiple linear regression analysis to determine the effect of measured serum cytokine concentrations as predictors of tryptophan metabolites was performed on inverse normal-rank transformations of the data, adjusted for sex, body mass index, smoking, alcohol intake, and contraceptive use in women.

Results: Median serum CRP and neopterin concentrations were well below established clinical cutoffs for inflammation. We observed significant positive associations between serum interleukin-10 (IL-10) and serum kynurenine (P = 0.0002), the kynurenine-to-tryptophan ratio (KTR) (P = 0.003), 3-hydroxykynurenine (P = 0.01), and 3-hydroxyanthranilic acid (P = 0.04). Serum neopterin was positively associated with kynurenine, the KTR (both P < 0.0001), and anthranilic acid (P = 0.004), and was negatively associated with serum tryptophan (P = 0.01) and PLP (P < 0.0001). Serum tumor necrosis factor α was also negatively associated with tryptophan (P < 0.001).

Conclusions: In healthy young adults with no apparent inflammatory conditions, serum tryptophan metabolites are significantly associated with key immune system biomarkers. The observed association between IL-10 and kynurenine is unexpected and suggests that kynurenine-linked mechanisms promoting negative regulation of inflammatory responses are associated with normal immune homeostasis. *J Nutr* 2016;146:1801–6.

Keywords: tryptophan, kynurenine, cytokines, neopterin, interleukin-10

Introduction

Activation of the immune system results in accelerated tryptophan catabolism through the kynurenine pathway. In the liver, the first step of tryptophan catabolism is catalyzed by tryptophan 2,3-dioxygenase, a constitutive enzyme that is inducible by stress hormones and is regulated by the availability of its substrate, L-tryptophan. Indoleamine 2,3-dioxygenase (IDO)[11] 1 is an extrahepatic enzyme with equivalent action in tryptophan

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[3] Supplemental Tables 1–3 are available from the "Online Supporting Material" link in the online posting of the article and from the same link in the online table of contents at http://jn.nutrition.org.
*To whom correspondence should be addressed. E-mail: amolloy@tcd.ie.
[11] Abbreviations used: CRP, C-reactive protein; IDO, indoleamine 2,3-dioxygenase; KTR, kynurenine-to-tryptophan ratio; PLP, pyridoxal 5′-phosphate; TSS, Trinity Student Study.

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degradation. This enzyme has minimal activity in nonpathologic conditions, but is highly inducible by proinflammatory cytokines, especially IFNγ (1–3). A second isoform of IDO exists, IDO2, but it is thought to be present only in tumor cells and is not involved in tryptophan metabolism (4). IDO1 activation increases tryptophan catabolism, resulting in the formation of kynurenine metabolites and an increase in the kynurenine-to-tryptophan ratio (KTR). IFNγ also stimulates neopterin release from macrophages (5–7). The KTR and neopterin both are considered to be markers of immune activation mediated by IFNγ (8–10).

Tryptophan catabolism through the kynurenine pathway is highly dependent on vitamin B-6. Low plasma concentrations of the biologically active B-6 vitamer, pyridoxal 5′-phosphate (PLP), have been observed in patients with high blood concentrations of inflammatory markers, such as C-reactive protein (CRP), IL-6, neopterin, and the KTR (11, 12), and in patients with chronic inflammation (13, 14). However, it is not clear whether this is merely a reflection of altered distribution of PLP between intra- and extracellular compartments during inflammation (13, 15), or if an increased PLP requirement during inflammation leads to low PLP status (16).

Induction of IDO1 by inflammatory signals alters the inflammatory process not only by tryptophan depletion, but also by the formation of kynurenine metabolites that have immunomodulatory effects (17, 18). Kynurenine, kynurenic acid, and xanthurenic acid, for example, reduce inflammation by limiting the production of IFNγ by immune cells (19). In addition, kynurenine metabolites are involved in immune tolerance and T cell modulation (20, 21). Because of these close interactions, we hypothesized that even in the absence of clinically relevant inflammation, a metabolic balance would exist between these diverse immunomodulatory metabolites that would be demonstrated in a relation between serum kynurenine metabolites and circulating cytokines in healthy individuals. Therefore, we addressed this question in a large cohort of young healthy adults with no clinically apparent inflammatory conditions.

Methods

Subjects. The Trinity Student Study (TSS) enrolled students attending the University of Dublin, Trinity College, between February 2003 and February 2004. Eligibility criteria included age between 18 and 28 y, Irish ethnicity based on origins of grandparents, and no current serious medical condition. A total of 2524 subjects were eligible to participate and were requested to donate a blood sample and complete a detailed lifestyle questionnaire. The study was conducted according to the guidelines laid down in the Declaration of Helsinki. Ethical approval was obtained from the Dublin Federated Hospitals Research Ethics Committee, which is affiliated with the University of Dublin, Trinity College, and subjects gave written informed consent. The study was reviewed by the Office of Human Subjects Research at the US NIH. Further details have been published elsewhere (22, 23). Fifteen subjects with no questionnaire data and one duplicate sample were excluded, leaving 2508 valid participants. Because of funding constraints, cytokine analysis could be carried out only on 800 randomly selected subjects from the full TSS cohort. Tryptophan and its metabolites, vitamin B-6, and lifestyle data, including smoking status, reported alcohol intake, and contraceptive use in women, were available on the full set of 800 subjects. Average alcohol consumption, converted to grams of ethanol per day, was categorized into never or occasional (0 to <15 g/d), light to moderate (15 to <30 g/d); and heavy (≥30 g/d).

Blood collection and biochemical analyses. Nonfasting blood samples were collected on the day of the interview. Blood was collected into serum and EDTA-coated plasma tubes, kept cool, then separated within 3 h of collection and stored at −80°C until analyzed. Plasma vitamin B-6 species and serum tryptophan metabolites plus neopterin and CRP were measured with the use of high-throughput LC–tandem MS assays in the laboratory at Bevital (www.bevital.no). Interassay CVs were <16.9% for all metabolites (24). Serum-based liver and kidney function tests were carried out commercially by Claymon Laboratories, Dublin, Ireland, with the use of an automated Abbott architect auto-analyzer platform with standard kits. Inter- and intra-assay CVs were <4.8%. Serum cytokine concentrations, including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, and IL-10, in addition to IFNγ, TNFα, and monocyte chemoattractant protein 1, were measured with the use of a high-sensitivity ELISA kit (Randox Laboratories). The sensitivity of the assays ranged from 0.12 pg/mL for IL-6 to 2.12 pg/mL for IL-4. The intra-assay and interassay precision expressed, as percentage CVs, were ≤12.2 (25). All samples were analyzed for the metabolites in large batches after the conclusion of recruitment. We did not observe any significant trends in the data over the period of study that we could attribute to increased periods of infection or laboratory-based drifts in analysis. Concentrations of some cytokines and inflammatory markers were below the limit of detection in the following numbers of individuals—CRP: $n = 52$; IL-1α: $n = 9$; IL-1β: $n = 45$; IL-2: $n = 27$; IL-4: $n = 2$; IL-10: $n = 1$; IFNγ: $n = 11$; and TNFα: $n = 1$. Based on previous reports, serum CRP >10 mg/L and neopterin >10 nmol/L were considered to be clinically relevant indicators of current inflammation (26–28). In an effort to eliminate individuals with possible subclinical inflammation, we removed 47 subjects who had concentrations of CRP >10 mg/L or neopterin >10 nmol/L. Furthermore, although there are no clinical reference guidelines for any of the other cytokines in relation to inflammatory processes, we removed any subject who had an extreme outlier at the upper end of the distribution for >2 of the 10 remaining cytokines (additional to CRP and neopterin). We could not use the SD as an objective basis for defining an outlier, because all of the cytokines had very non-normal distributions. We therefore chose the IQR as the guideline and defined an extreme outlier with the use of the following formula: 75th percentile + (7 × IQR). This resulted in the removal of a further 16 subjects. The final data analysis was therefore carried out on 737 subjects.

Statistical analysis. We performed all statistical analyses with the use of SAS software, version 9.3. The cytokines, vitamin B-6 vitamers, and tryptophan metabolites had non-normal distributions, so data are expressed as median (range: 5th, 95th percentile). For comparisons between sexes, we used Student's Independent t tests or Mann-Whitney U tests for continuous data and we used chi-square tests to compare differences in categorical data. Correlation analysis was performed with the use of Spearman correlation coefficients. Inverse normal-rank transformations were applied to the cytokines, tryptophan metabolites, KTR, and PLP to make them satisfy the normality assumption before examination of associations with any of the cytokines through multiple linear regression analysis. In separate regression models, we selected each of the tryptophan metabolites and PLP as dependent variables. The inverse normal rank transformed data were regressed on each cytokine for association analysis while adjusting for sex, BMI, smoking, alcohol intake, and oral contraceptive use. Statistical significance was set at $P < 0.05$.

Results

Characteristics of the study population. The general characteristics of participants in this study are shown in **Table 1**. A total of 62.7% of the subjects were women. Liver and kidney function tests were within clinically normal reference ranges (29). The concentrations of tryptophan and its kynurenine metabolites in the full TSS cohort have been previously reported (22). Details of these metabolites in the subset of the TSS that were included in the current study are provided in **Supplemental Table 1**. There were no significant differences between the tryptophan metabolite concentrations in the subset studied here and the total TSS cohort (Supplemental Table 1).

Distributions of immune system biomarkers. Data for serum cytokines and inflammation biomarkers are presented in

TABLE 1 General and lifestyle characteristics of healthy young adults in the TSS for whom serum cytokine measurements were available[1]

Variable	Total (n = 737)	Women (n = 462)	Men (n = 275)
Age,[2] y	22 (20, 25)	22 (19, 25)	23 (20, 26)**
BMI,[2] kg/m^2	22.4 (19.0, 28.3)	22.2 (18.8, 28.7)	22.9 (19.6, 28.2)*
Serum tests for kidney and liver function[2]			
Creatinine, μmol/L	63.9 (46.7, 87.7)	58.4 (44.5, 73.4)	74.1 (57.7, 94.8)***
Uric acid, μmol/L	257 (156, 410)	219 (147, 325)	330 (229, 443)***
Urea, μmol/L	4.3 (2.9, 6.6)	4.0 (2.7, 5.6)	5.0 (3.4, 7.1)***
γ-glutamyltransferase, IU/L	15.0 (9.0, 35.7)	14.0 (9.0, 26.3)	17.9 (12.0, 43.7)***
Alanine aminotransferase, IU/L	14.0 (8.00, 29.7)	13.0 (8.0, 23.0)	17.0 (10.0, 36.4)***
Smoking in previous 4 mo[3]	217 (29.5)	125 (27.1)	92 (33.6)
Alcohol[3]			
Never or occasional	287 (38.9)	232 (50.2)	55 (20.0)***
Moderate	247 (33.5)	155 (33.5)	92 (33.5)
Heavy	203 (27.5)	75 (16.2)	128 (46.5)***

[1] Values are medians (5th, 95th percentiles) or n (%). The TSS included 2508 participants. Subjects included in the current study were from a random subset of 800 TSS participants on whom cytokine analysis was performed. Subjects with C-reactive protein >10 mg/L or neopterin >10 nmol/L or with multiple (>2) cytokines >7 times the IQR were excluded as having possible subclinical inflammation, leaving a final set of 737 subjects for data analysis. *,**,***Different from women: *$P < 0.05$, ** $P < 0.001$, ***$P < 0.0001$. TSS, Trinity Student Study.
[2] Student's independent t tests were used to test differences between log-transformed continuous variables.
[3] Chi-square tests were used to test differences between unadjusted categorical variables.

Table 2. There were small but significant sex differences for some measured biomarkers. Men had higher concentrations of TNFα. Women had higher concentrations of the majority of ILs. In this healthy young cohort, the median concentration of both CRP and neopterin were well below the clinical cutoff limits for inflammation (28). Spearman correlations between all the measured immune system markers in these subjects can be seen in **Supplemental Table 2.**

Associations of immune system biomarkers with kynurenine metabolites. In separate models, we selected each of the tryptophan metabolites and PLP as dependent variables and performed multiple linear regression analysis against immune system biomarkers as independent variables, adjusting for sex, BMI, smoking, alcohol intake, and oral contraceptive use. The immune system biomarkers that showed significant associations with tryptophan metabolites are reported in **Table 3.** The biomarker most commonly associated with tryptophan metabolites was neopterin, which had positive associations with the KTR, kynurenine (both $P < 0.0001$), and anthranilic acid ($P = 0.004$). Neopterin also was negatively associated with tryptophan ($P = 0.01$) and PLP ($P < 0.0001$). Surprisingly, IL-10 was

TABLE 2 Effects of sex on serum concentrations of inflammation biomarkers and cytokines in a subset of healthy young adults from the TSS[1]

Variable	Total (n = 737)	Women (n = 462)	Men (n = 275)
Inflammation biomarkers[2]			
Kynurenine-to-tryptophan ratio, nmol/L:μmol/L	19.6 (14.0, 27.2)	19.3 (13.9, 26.3)	20.2 (14.4, 28.9)
Neopterin, nmol/L	3.79 (2.10, 7.97)	3.94 (2.10, 7.92)	4.03 (2.03, 8.18)
C-reactive protein, mg/L	0.51 (0.0, 4.66)	0.55 (0.0, 5.16)	0.44 (0.0, 3.21)
Cytokines,[2] ng/L			
IFNγ	0.56 (0.25, 2.06)	0.54 (0.25, 2.02)	0.60 (0.25, 2.07)
IL-2	1.33 (0.89, 3.33)	1.37 (0.88, 3.68)	1.23 (0.0, 2.70)***
IL-4	2.00 (1.33, 4.14)	2.05 (1.37, 4.42)	1.95 (1.32, 3.56)*
IL-6	0.79 (0.38, 2.25)	0.82 (0.39, 2.24)	0.74 (0.36, 2.28)**
IL-8	8.06 (3.92, 20.2)	8.48 (4.08, 22.2)	7.47 (3.64, 18.2)**
IL-10	0.68 (0.38, 1.88)	0.70 (0.40, 1.86)	0.64 (0.36, 2.00)*
IL-1α	0.17 (0.09, 0.85)	0.18 (0.09, 0.91)	0.15 (0.09, 0.58)***
IL-1β	1.39 (0.0, 4.40)	1.51 (0.13, 4.86)	1.29 (0.0, 3.48)***
MCP-1	193 (93.4, 348)	189 (91.8, 332)	197 (100, 368)
TNFα	4.60 (2.46, 13.04)	4.44 (2.27, 11.8)	4.87 (2.73, 14.9)*

[1] Values are medians (5th, 95th percentiles). The TSS included 2508 participants. Subjects included in the current study were from a random subset of 800 TSS participants on whom cytokine analysis was performed. Subjects with C-reactive protein >10 mg/L or neopterin >10 nmol/L or with multiple (>2) cytokines >7 times the IQR were excluded as having possible subclinical inflammation, leaving a final set of 737 subjects for data analysis. Some cytokines had measured concentrations under the lower limit of detection and were assigned a value of 0.0 for statistical analysis. These included C-reactive protein (n = 52), IL-1α (n = 9), IL-1β (n = 45), IL-2 (n = 27), IL-4 (n = 2), IL-10 (n = 1), IFNγ (n = 11), and TNFα (n = 1). *,**,*** Different from women: *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. MCP-1, monocyte chemoattractant protein 1; TSS, Trinity Student Study.
[2] Mann-Whitney U tests were used to test differences between variables.

TABLE 3 The contribution of selected serum inflammation biomarker and cytokine concentrations as independent predictors of tryptophan, its metabolites, and pyridoxal 5′-phosphate in a subset of healthy young adults from the TSS[1]

	Neopterin		IL-10		TNF-α		MCP-1		CRP	
Dependent variable	Estimate[2]	*P*	Estimate[2]	*P*	Estimate[2]	*P*	Estimate[2]	*P*	Estimate[2]	*P*
Kynurenine-to-tryptophan ratio	0.26	<0.0001	0.11	0.003	0.07	0.047	−0.05	0.22	0.06	0.12
Tryptophan	−0.10	0.012	0.01	0.72	−0.12	<0.001	0.11	0.003	−0.05	0.21
Kynurenine	0.17	<0.0001	0.13	0.0002	−0.04	0.31	0.06	0.09	0.02	0.63
3-Hydroxykynurenine	0.10	0.026	0.10	0.012	0.05	0.25	−0.01	0.76	0.08	0.08
Kynurenic acid	0.07	0.08	0.00	0.92	−0.05	0.18	0.08	0.031	−0.06	0.15
Xanthurenic acid	−0.05	0.25	0.02	0.61	−0.03	0.46	0.06	0.13	0.01	0.73
Anthranilic acid	0.11	0.004	0.05	0.17	−0.06	0.11	0.04	0.23	−0.03	0.49
3-Hydroxyanthranilic acid	−0.01	0.78	0.08	0.043	0.00	0.91	0.04	0.33	0.09	0.039
Pyridoxal 5′-phosphate	−0.16	<0.0001	−0.03	0.46	−0.03	0.39	0.00	0.96	−0.11	0.005

[1] The TSS included 2508 participants. Subjects included in the current study were from a random subset of 800 TSS participants on whom cytokine analysis was performed. Subjects with CRP >10 mg/L or neopterin >10 nmol/L or with multiple (>2) cytokines >7 times the IQR were excluded as having possible subclinical inflammation, leaving a final set of 737 subjects for data analysis. CRP, C-reactive protein; MCP-1, monocyte chemoattractant protein 1; TSS, Trinity Student Study.

[2] For regression analysis, all tryptophan metabolites and cytokines were normalized by inverse normal-rank transformations. Pyridoxal 5′-phosphate and each of the tryptophan metabolites were treated as dependent variables in separate multinomial regression models, in which they were regressed on the measured cytokines for independent association and adjusted for sex, BMI, smoking, alcohol intake and oral contraceptive use. Statistical significance was set at $P < 0.05$.

the next most commonly associated immune system biomarker, being positively associated with 4 tryptophan-related variables: kynurenine ($P = 0.0002$), the KTR ($P = 0.003$), 3-hydroxykynurenine ($P = 0.01$), and 3-hydroxyanthranilic acid ($P = 0.04$). Serum TNFα was negatively associated with tryptophan ($P < 0.001$), but was not significantly associated with any of its kynurenine metabolites. CRP was negatively associated with PLP ($P = 0.005$), and monocyte chemoattractant protein 1 was positively associated with tryptophan ($P = 0.003$). Neither IFNγ nor any of the other measured ILs were associated with any of the tryptophan metabolites measured (**Supplemental Table 3**).

Discussion

To our knowledge, this is the first study to explore the relation between tryptophan metabolites and multiple immune system biomarkers in a large cohort of healthy adults with no apparent inflammatory conditions. We showed that, even in a young healthy population, intermediates of vitamin B-6–dependent tryptophan catabolism were clearly related to key immune system biomarkers, such as neopterin and IL-10. For example, neopterin had a significant positive association with the KTR and a negative association with PLP. These relations have been reported in inflammatory conditions (30, 31) and in older subjects (32); we showed that they are also present in young healthy adults with minimal evidence of inflammation.

Surprisingly, we found that IL-10 had a highly significant positive association with kynurenine ($P = 0.0002$) in these young adults. One previous study reported a positive association between kynurenine and IL-10 in cerebral spinal fluid; however, this was in patients with bacterial meningitis (33), and, to the best of our knowledge, this has never been reported in a healthy population with little or no evidence of inflammatory disease. One possible mechanism by which this might occur is via the aryl hydrocarbon receptor (20, 34). In this scenario, IDO1 activation and enhanced kynurenine production could lead to binding of kynurenine to the aryl hydrocarbon receptor, which is important for the differentiation of naive T cells into highly potent CD25+Foxp3+ regulatory T cells (20). In turn, CD25+Foxp3+ regulatory T lymphocytes are strong inducers of IL-10, which is a central anti-inflammatory cytokine. This mechanism would allow kynurenine to exert a negative feedback loop on an initial acute inflammatory event and maintain an ongoing balance between inflammation and immunosuppression activity.

Circulating PLP concentrations are lower in inflammatory states, and it is reported that PLP-dependent reactions of the kynurenine pathway are involved in the immune response by suppressing inflammation, reducing the tissue damage caused by the immune response, and developing immune tolerance (35–37). We showed that, even in a young healthy population, PLP has significant negative correlations with neopterin and CRP. It is believed currently that the inverse relation between PLP and inflammation is a result of mobilization of PLP to the inflammation site to be used by PLP-dependent enzymes that are involved in the inflammatory response (13, 31, 38). Moreover PLP depletion during inflammation may reflect the accelerated synthesis of cytokines (39) and the activation and proliferation of lymphocytes (39, 40), both of which are key events in the inflammatory process. It has also been proposed that the lower PLP concentrations found in inflammation could reflect a higher demand and uptake of PLP at the site of inflammation for degradation of tryptophan via the kynurenine pathway, which is heavily dependent on PLP (38).

CRP is one the most commonly measured inflammatory markers, because its synthesis is induced by inflammatory cytokines, such as IL-6 and TNFα (35). CRP concentrations previously were shown to have a negative correlation with PLP in both healthy and disease states (16, 38) and positive correlations with the KTR in ischemic stroke patients (41). In this study, CRP was not associated with tryptophan metabolites in healthy subjects after adjustment for relevant confounding factors.

We have previously reported the effect of smoking, alcohol intake and oral contraceptive use on tryptophan metabolism (22). Because these lifestyle factors are well known to have an effect on inflammatory processes, it was important to adjust the regression models for them. Although tryptophan metabolites

are generally higher in men than in women (22), most of the measured ILs were higher in women. Other studies showed that, with in vitro stimulation, cytokine concentrations varied with hormonal status (42, 43). It is believed that sex differences largely are the result of the immune-enhancing effects of estrogens and immunosuppressive effects of androgens and progesterone (44), giving women a stronger humoral immune response, but also a higher susceptibility to autoimmunity. In this cross-sectional study, we did not collect information that might enable us to assess the effect of the menstrual cycle on these cytokines.

One strength of this study is that it was based on a large cohort of healthy adults, and not on a diseased population in which many factors underlie the inflammatory condition. In addition, we included a wide variety of measured immune system biomarkers. One limitation was that cytokine concentrations in some individuals were below the limit of detection. We used a standard statistical approach, designating them as zero, to take consideration of these factors. As with most other studies on tryptophan metabolism, this is an association study, which does not provide causal evidence.

In conclusion, this study demonstrated that several key relations between cytokines and tryptophan metabolites reported in inflammatory conditions are also present in healthy young adults without acute or chronic inflammatory disease. Our unexpected observation of significant associations between the anti-inflammatory cytokine IL-10 and kynurenine metabolites indicates that interactions between tryptophan catabolism and immune system cytokines occur even in the presence of minimal inflammation and suggests that this pathway may serve as a mechanism to promote negative regulation as part of normal immune homeostasis.

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