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# Blood Folate Status and Expression of Proteins Involved in Immune Function, Inflammation, and Coagulation: Biochemical and Proteomic Changes in the Plasma of Humans in Response to Long-Term Synthetic Folic Acid Supplementation

**Susan J. Duthie,*,† Graham Horgan,‡ Baukje de Roos,† Garry Rucklidge,† Martin Reid,† Gary Duncan,† Lynn Pirie,† Graham P. Basten,§ and Hilary J. Powers§**

*Aberdeen University Rowett Institute of Nutrition and Health, Aberdeen, U.K., Biomathematics & Statistics Scotland (BioSS), Aberdeen, U.K., Human Nutrition Unit, Division of Clinical Sciences (North), The University of Sheffield, Sheffield, U.K.*

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We used plasma proteomics to identify human proteins responsive to folate status. Plasma was collected from subjects treated with placebo or 1.2 mg of folic acid daily for 12 weeks in a randomized controlled trial. Homocysteine and folate were measured by immunoassay and uracil misincorporation by electrophoresis. The plasma proteome was assessed by 2-D gel electrophoresis, and proteins were identified by LC MS/MS. 5-methylTHF increased 5-fold ($P = 0.000003$) in response to intervention. Red cell folate doubled ($P = 0.013$), and lymphocyte folate increased 44% ($P = 0.0001$). Hcy and uracil dropped 22% ($P = 0.0005$) and 25% ($P = 0.05$), respectively. ApoE A-1, α-1-antichymotrypsin, antithrombin, and serum amyloid P were downregulated, while albumin, IgM C, and complement C3 were upregulated ($P < 0.05$). More than 60 proteins were significantly associated with folate pre- and postintervention ($P < 0.01$). These were categorized into metabolic pathways related to complement fixation (e.g., C1, C3, C4, Factor H, Factor 1, Factor B, clusterin), coagulation (e.g., antithrombin, α-1-antitrypsin, kininogen) and mineral transport (e.g., transthyretin, haptoglobin, ceruloplasmin). Low folate status pre- and post-treatment were associated with lower levels of proteins involved in activation and regulation of immune function and coagulation. Supplementation with synthetic folic acid increased expression of these proteins but did not substantially disrupt the balance of these pathways.

**Keywords:** synthetic folic acid supplementation • human study • folate status • plasma proteomics • immune function

## Introduction

Folates are a family of water-soluble B vitamins that act as carbon donors in the synthesis of purines and thymidylate for DNA synthesis and repair and influence gene expression through deoxycytosine methylation. Folates also donate 1-C units for the remethylation of homocysteine to methionine, which is converted to *S*-adenosylmethionine (SAM), the principal methyl donor in cellular methylation and protein synthesis.[1] Severe folate deficiency causes megaloblastic anemia in adults and congenital defects in the newborn.[2] Poor folate status is also a risk factor for heart disease[3] and cognitive dysfunction and dementia in the elderly.[4] Folate deficiency has been implicated in the development of cancer, notably of the cervix, lung, breast, brain, and colon.[5,6] Folate deficiency affects a substantial percentage of the population, notably adolescents, the institutionalized elderly, and people in low-socioeconomic groups.

Evidence for a protective effect of periconceptional synthetic folic acid supplementation against neural tube defects (NTDs) prompted introduction of mandatory fortification of flour and uncooked cereal-grain products in the U.S. and Canada in 1998. Simultaneously, Western Australia promoted voluntary fortification of foodstuffs with folate.

The effect of fortification on blood folate status has been dramatic. Plasma, serum, and whole blood folate measured in the Framingham Offspring Study birth cohort and across several waves of the NHANES cross-sectional population surveys increased and plasma total homocysteine decreased significantly.[7,8] Serum folate has increased 38%, and homocysteine has fallen 21% in Australia.[9] Preliminary reports indicate a significant reduction in the incidence of NTDs in those countries that adopted fortification.[10−14]

The U.K. and many other European governments have still to decide whether to introduce mandatory folic acid fortification. The potential benefit of increasing folate intake popula-

* To whom correspondence should be addressed at the Nutrition and Epigenetics Group, Division of Vascular Health, Aberdeen University Rowett Institute of Nutrition and Health, Greenburn Road, Bucksburn, Aberdeen AB21 9SB, U.K. Tel: +44 1224 712751Ext. 2324. Fax: +44 1224 716629. E-mail: sd@rri.sari.ac.uk.

† Aberdeen University Rowett Institute of Nutrition and Health.

‡ Biomathematics & Statistics Scotland (BioSS).

§ The University of Sheffield.

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tion-wide and the positive impact this may have on other aspects of human health is unknown. Conversely, there is concern that substantially increasing synthetic folate intake may have adverse effects in certain groups of individuals. The level of folate fortification in the U.S. was determined on the basis that it would not increase intakes in excess of the upper safe limit of 1 mg per day in any population group.[15] The actual increase in folate intake may be higher than anticipated.[16,17] Potential adverse effects of high circulating levels of free folic acid include masking of pernicious anemia in the elderly and promotion of initiated cancer cells.[18,19] A recent study has reported that fortification may increase cognitive dysfunction in $B_{12}$-deficient subjects.[20]

We have carried out a human intervention study feeding folic acid (1.2 mg/day) for 12 weeks. We report how significantly increasing synthetic folic acid intake alters the plasma proteome in normal healthy subjects and how this is associated with changes in blood biomarkers of folate status.

## Experimental Procedures

**Study Subjects.** The present analysis was carried out on blood samples from a subset of volunteers who participated in a folic acid intervention study as previously reported.[21] Briefly, a randomized double-blind placebo-controlled folic acid intervention study was carried out in healthy subjects with red cell folate levels within the normal range. Volunteers were randomized to receive either 1.2 mg per day of folic acid or glucose placebo for 12 weeks. Sixty-one volunteers completed the original study (30 in the supplemented group and 31 in the placebo group). We have previously described the effect of folic acid supplementation on folate status and genomic stability in this group.[21,22]

The present study examines the consequences of increasing synthetic folic acid intake on global protein expression in plasma from a subset of these volunteers.

Plasma from 20 study subjects (10 placebo and 10 folic acid supplemented individuals) was selected (at random) for analysis. The supplement group consisted of 3 men and 7 women, with a mean age of 39.9 ± 3.5 years, and the placebo group consisted of 2 men and 8 women, 40.2 ± 2.6 years old. There were no significant differences in anthropometrical indices either between these groups or between this subset and the original study groups.[21,22]

**Preparation of Whole Blood, Plasma, and Erythrocytes for Measurement of Folate, 5-Methyltetrahydrofolate, Homocysteine, *S*-Adenosylhomocysteine, and Uracil Misincorporation.** This has been described in detail previously.[21] Briefly, volunteers donated a 20 mL fasted (16 h) blood sample into EDTA vacutainers at week 0 (baseline) and after 12 weeks on placebo or folic acid supplements. The hematocrit was recorded and an aliquot of whole blood stored for measurement of red cell folate (by chemiluminescence immunoassay; Abbot IMx analysis) and whole blood SAM (by HPLC). The remaining whole blood sample was centrifuged at 2,400*g* at 4 °C for 15 min and the plasma layer stored for analysis of total homocysteine (by immunoassay as above) and 5-methyltetrahydrofolate (5-methylTHF; by HPLC) and for plasma proteomics analysis (see below). All methods, together with measures of intra- and interassay variability, have been described in detail elsewhere.[21] The lymphocyte-containing "buffy coat" was removed for analysis of folate concentrations[21] and uracil misincorporation by single-cell gel electrophoresis.[22]

**Preparation of Plasma for Proteomic Analysis.** Plasma samples were depleted of the 12 most abundant proteins (albumin, IgG, fibrinogen, transferrin, IgA, IgM, HDL Apo AI, HDL Apo AII, haptoglobulin, α1-antitrypsin, α1-acid glycoprotein, and α2-macroglobulin) by running 20 μL aliquots of plasma through an IgY-12 high-capacity proteome partitioning kit (ProteomeLab, Beckman Coulter) according to the manufacturer's instructions. The study plasma samples (for single volunteers) were run sequentially through the columns in the order (1) placebo baseline, (2) placebo 12 weeks, (3) folate-treated baseline, and (4) folate-treated 12 weeks, in order to minimize experimental variation due to column efficiency. The flow-through (0.5 mL) from each run was pooled to a final volume of 1.5 mL. Samples (1.0 mL) were concentrated to 100 μL using Millipore Ultrafree-0.5 centrifugal spin columns and rehydration buffer (7 M urea, 2 M thiourea, 2% CHAPS and 0.5% IPG buffer, pH 4−7) added to a final volume of 150 μL. Protein concentration was quantified as described previously.[23] A 200 μg portion of depleted plasma protein sample was subsequently loaded per 17 cm 2-D gel. Optimal protocols for human plasma depletion, protein concentration, and 2-D gel electrophoresis have been described previously.[23]

**Proteomic Analyses: 2D-Gel Electrophoresis of Depleted Plasma Samples.** One 2-D gel was run per single plasma sample ($n = 10$ subjects per treatment group at each time point; 40 gels in total). Proteins were separated by isoelectric focusing in the first dimension (BioRad immobilized pH gradient (IPG) strips (pI range 4−7)) and SDS-PAGE in the second dimension on 8−16% acrylamide gels (18 × 18 cm). Gels were stained with Flamingo Red (BioRad) and imaged on a BioRad GS710 flat bed imager followed by image analysis using PD Quest Version 8.0.1. Spots were excised from the SDS-PAGE gels using a robotic BioRad spot cutter, trypsinized in a MassPrep station (Waters, MicroMass, Manchester, U.K.) and analyzed by LC-MS/MS using an Ultimate nano LC capillary chromatography system (LC Packings, Camberly, Surrey, U.K.) combined with an Applied Biosystems 4000 Q-Trap (Warrington, U.K.). Peptide fragment mass spectra yielded the sequence of separated peptides that were pasted into the fingerprinting web resource program "Mascot" (Matrix Science Ltd., Boston, MA) for protein identification.[23,24]

**Statistics.** For proteomic analysis, gel images were examined using PDQuest software (Biorad). After normalization and matching, spot densities were exported for multivariate data analysis using Excel and Genstat (version 11). Log transformed spot densities calculated as change in spot density (between weeks 0 and 12) for all 20 subjects irrespective of intervention, were analyzed initially by Student's *t* test at 5% significance ($P < 0.05$) to determine treatment effects. Analyses of associations between blood folate status markers and plasma proteins (1) preintervention, (2) after 12 weeks of treatment, and (3) due to change for all 20 volunteers were carried out using Pearson correlation coefficients within Genstat. Spots with densities that either differed significantly due to intervention ($p < 0.05$) or were highly correlated ($P < 0.01$) with folate status (pre- and postintervention), were cut from the gel and identified by LC MS/MS. For analyses of blood biochemistry and uracil misincorporation, unpaired Student's *t* test was carried out using Excel.

**Table 1.** Baseline (Week 0) and Postintervention (Week 12) Folate Status Biochemical Biomarkers[a]

| | placebo | | folic acid | | | |
|---|---|---|---|---|---|---|
| biomarker | week 0 | week 12 | week 0 | week 12 | difference (95% CI) | *P* |
| 5-methylTHF (nmol/L) | 20.49 (2.04) | 20.61 (1.58) | 15.11 (1.58) | 92.49 (11.71) | 77.26 (56.82−98.19) | 0.000003 |
| red cell folate (nmol/L) | 458.15 (20.94) | 662.30 (112.64) | 529.20 (22.21) | 1111.66 (113.96) | 405.30 (56.31−754.29) | 0.013 |
| lymphocyte folate (ng/$10^6$ cells) | 0.28 (0.04) | 0.25 (0.04) | 0.23 (0.04) | 0.56 (0.08) | 0.36 (0.19−0.52) | 0.00011 |
| homocysteine ($\mu$mol/L) | 12.26 (1.20) | 13.62 (1.52) | 12.23 (0.71) | 10.06 (0.61) | −3.39 (−5.21 to −1.56) | 0.00048 |
| SAM ($\mu$mol/L) | 0.33 (0.29) | 0.20 (0.17) | 0.28 (0.17) | 1.67 (0.71) | 1.19 (−0.21 to +2.58) | 0.079 |
| uracil (arbitrary units) | 54.2 (4.7) | 52.8 (4.6) | 52.7 (3.0) | 39.7 (2.7) | −10.25 (−24.62 to +4.12) | 0.045 |

[a] Values are mean with SEM in parentheses for both placebo and intervention groups pre- and postintervention ($n = 10$ per group, with the exception of SAM, where $n = 8$ for folic acid treated subjects and $n = 5$ for placebo-treated subjects). The response to folic acid supplementation was calculated by subtracting baseline values from treatment values in all subjects (difference with 95% CI). *P* values refer to significant differences between change in the intervention group and change in the placebo group using unpaired Student's *t* test.

**Table 2.** Human Plasma Proteins Significantly Up- or Downregulated due to Supplementation with Folic Acid[a]

| | placebo | | folic acid | | | | |
|---|---|---|---|---|---|---|---|
| spot | week 0 | week 12 | week 0 | week 12 | difference (95% CI) | *P* | protein |
| 1 | 1.88 (0.33) | 2.19 (0.34) | 2.05 (0.29) | 2.03 (0.27) | −0.33 (−0.58, −0.08) | 0.019 | apolipoprotein A-I |
| 402 | 2.95 (0.52) | 3.10 (0.19) | 3.16 (0.23) | 2.89 (0.40) | −0.41 (−0.79, −0.04) | 0.018 | α-1-antichymotrypsin |
| 5302 | 1.91 (0.32) | 2.52 (0.75) | 2.04 (0.59) | 1.88 (0.43) | −0.76 (−1.44, −0.08) | 0.017 | antithrombin-III |
| 7102 | 2.45 (0.22) | 2.54 (0.38) | 2.64 (0.30) | 2.39 (0.41) | −0.34 (−0.69, −0.01) | 0.033 | serum amyloid P-component |
| 8105 | 2.36 (0.48) | 2.0 (0.41) | 2.09 (0.34) | 2.22 (0.38) | 0.49 (0.09, 0.89) | 0.021 | serum albumin |
| 8502 | 3.52 (0.47) | 3.18 (0.51) | 3.45 (0.31) | 3.43 (0.16) | 0.32 (0.04, 0.59) | 0.026 | IgM c chain |
| 8603 | 3.45 (0.29) | 3.14 (0.43) | 3.36 (0.28) | 3.44 (0.18) | 0.39 (0.03, 0.74) | 0.029 | IgM c chain |
| 9605 | 3.21 (0.15) | 2.88 (0.67) | 3.34 (0.52) | 3.24 (0.34) | 0.73 (−0.03, 1.48) | 0.038 | Complement C3 |
| 9702 | 3.17 (0.29) | 2.91 (0.57) | 2.47 (0.62) | 3.31 (0.16) | 1.10 (0.48, 1.72) | 0.006 | Complement C3 |

[a] Values are log transformed mean spot densities with SEM in parentheses for both placebo and intervention groups pre- and postintervention ($n = 10$ per group), as determined using PDQuest software. The response to folic acid supplementation was calculated by subtracting baseline values from treatment values in all subjects (difference with 95% CI). *P* values refer to significant differences between change in the intervention group and change in the placebo group using unpaired Student's *t* test.

## Results

**Blood Biomarkers of Folate Status and Genomic Stability.** Supplementation with 1.2 mg of folic acid per day significantly increased plasma 5-methyltetrahydrofolate and red cell and lymphocyte folate (approximately 500%, 110%, and 43%, respectively; Table 1). Conversely, total plasma homocysteine significantly decreased in response to folic acid intervention (approximately 22%; Table 1). Whole blood SAM concentrations were increased in the supplemented group, but not significantly ($P = 0.079$). Uracil misincorporation decreased in lymphocytes from supplemented volunteers (approximately 25%; Table 1). Folate status and uracil concentrations were unaltered in subjects taking placebo for 12 weeks (Table 1; $P > 0.05$ for all biomarkers). These data, showing the effects of folic acid intervention on biochemical markers of folate status in 20 subjects, agrees with that generated in the larger study.[21,22]

**Plasma Proteomics and Associations with Folate Status and DNA Stability.** Comparative proteomics revealed approximately 300 plasma spots that matched across all 40 gels. Spots that differed significantly ($P < 0.05$) due to folic acid supplementation (expressed as change in density (week 12 spot densities minus baseline spot densities) for each of the 20 subjects) were selected for cutting and identification. In addition, spots significantly associated ($P < 0.01$; $q = 0.65$) with changes in blood folate (plasma, lymphocyte, and whole blood total folate and plasma 5-methylTHF), plasma homocysteine and SAM concentrations, and uracil misincorporation both pre- and postintervention were identified.

A total of 62 spots (corresponding to 30 different proteins) that were influenced by folate status (reported either as change in protein expression due to supplementation or as being highly correlated with folate status pre- and postintervention) could be identified by LC MS/MS. Seven plasma proteins differed significantly by treatment after folate supplementation ($P < 0.05$; Table 2). Of these, four proteins were significantly downregulated in the folic acid intervention group (apolipoprotein A1, α-1-antichymotrypsin, antithrombin III, and serum amyloid P) and three proteins were significantly upregulated (serum albumin, IgM C chain, and complement protein C3).

Correlation analysis revealed more than 60 spots that were highly positively and negatively associated with baseline folate ($P < 0.01$; Table 3) and changes in folate status due to long-term folic acid supplementation ($P < 0.01$, mean $q = 0.65$; Tables 4 and 5). The effect of change in folate status on expression of proteins involved in immune function and ligand transport is shown in Figure 1. The strength of association between folate status and expression (for four sample proteins) is shown in Figure 2.

Proteins significantly associated with folic acid status (pre- and postintervention) were broadly categorized according to function (Table 6). Major metabolic pathways related to immune function, including complement activation and regulation (e.g., C1, C3, C4, factor H, factor 1, factor B, and clusterin; Figure 3), coagulation and fibrinolysis (e.g., antithrombin, α-1-antithrypsin, kininogen), regulation of vasoconstriction, vasodilation, and osmotic blood pressure (e.g., factor B), and vitamin and mineral transport and metabolism (e.g., transthyretin, haptoglobin, ceruloplasmin) were highly correlated with folate, Hcy, and SAM blood concentrations.

Measures of confidence for the 62 spots (30 proteins) that were successfully identified are presented in Table 6.

**Table 3.** Correlation Analysis of Baseline Folate Status and Human Plasma Proteins (Week 0)[a]

| blood biomarker | spot | protein | correlation | *P* |
|---|---|---|---|---|
| plasma 5-methylTHF | 5202 | serum albumin | 0.583 | 0.0070 |
| plasma 5-methylTHF | 7303 | heavy chain of factor I | 0.657 | 0.0016 |
| plasma 5-methylTHF | 7405 | serum albumin | 0.588 | 0.0063 |
| plasma 5-methylTHF | 8602 | complement factor B | 0.604 | 0.0048 |
| lymphocyte folate | 3006 | serum amyloid P-component | 0.686 | 0.0008 |
| plasma 5-methylTHF | 4101 | haptoglobin | 0.565 | 0.0094 |
| lymphocyte folate | 5501 | serum albumin | 0.629 | 0.0030 |
| lymphocyte folate | 5502 | serum albumin | 0.712 | 0.0004 |
| lymphocyte folate | 6503 | hemopexin | 0.592 | 0.0059 |
| red cell folate | 2101 | haptoglobin | 0.588 | 0.0064 |
| red cell folate | 7001 | haptoglobin | 0.592 | 0.0060 |
| red cell folate | 1503 | vitronectin | 0.562 | 0.0099 |
| plasma hcy | 3602 | afamin | 0.643 | 0.0024 |
| plasma SAM | 1203 | complement C3 | 0.601 | 0.0086 |
| plasma SAM | 1403 | kininogen-1 | 0.809 | 0.0001 |
| plasma SAM | 4501 | α-1B-glycoprotein | 0.739 | 0.0007 |
| plasma SAM | 7601 | serum albumin | 0.783 | 0.0002 |
| plasma SAM | 8404 | serum albumin | 0.791 | 0.0002 |
| plasma 5-methylTHF | 3101 | light chain of factor I | −0.594 | 0.0057 |
| plasma 5-methylTHF | 3104 | apolipoprotein A-IV | −0.685 | 0.0009 |
| plasma 5-methylTHF | 3501 | α-1B-glycoprotein | −0.556 | 0.0110 |
| plasma 5-methylTHF | 3602 | afamin | −0.582 | 0.0071 |
| lymphocyte folate | 5301 | antithrombin-III | −0.625 | 0.0032 |
| red cell folate | 9402 | Ig γ-1 chain C region | −0.636 | 0.0026 |
| plasma hcy | 3302 | vitamin D-binding protein variant | −0.649 | 0.0021 |

[a] Blood biomarker, spot number, protein name, and correlation strength (positive and negative) are shown together with the corresponding *P* value.

**Table 4.** Correlation Analysis of Folate Status and Human Plasma Proteins at the End of Treatment (Week 12)[a]

| blood biomarker | spot | protein | correlation | *P* |
|---|---|---|---|---|
| lymphocyte folate | 7604 | serum albumin | 0.578 | 0.0075 |
| red cell folate | 301 | α-2-HS-glycoprotein | 0.580 | 0.0073 |
| red cell folate | 1702 | WD repeat-containing protein 22 | 0.563 | 0.0097 |
| SAM | 5702 | complement factor H | 0.696 | 0.0017 |
| SAM | 6501 | hemopexin | 0.611 | 0.0075 |
| SAM | 7105 | IGKV1-5 | 0.682 | 0.0022 |
| SAM | 7604 | serum albumin | 0.888 | 0.0000 |
| SAM | 8105 | serum albumin | 0.665 | 0.0031 |
| SAM | 8110 | IgG κ chain | 0.592 | 0.0098 |
| SAM | 8303 | serum albumin | 0.613 | 0.0072 |
| SAM | 8304 | serum albumin | 0.702 | 0.0015 |
| plasma hcy | 1403 | kininogen-1 | 0.809 | 0.0000 |
| plasma hcy | 6105 | apolipoprotein A-I | 0.592 | 0.0063 |
| plasma hcy | 6301 | antithrombin III | 0.579 | 0.0079 |
| plasma hcy | 6804 | α-2-macroglobulin | 0.732 | 0.0003 |
| red cell folate | 4604 | ceruloplasmin | −0.615 | 0.0039 |
| red cell folate | 5601 | ceruloplasmin | −0.581 | 0.0072 |
| uracil misincorporation | 3502 | α-1B-glycoprotein | −0.586 | 0.0069 |

[a] Blood biomarker, spot number, protein name, and correlation strength (positive and negative) are shown together with the corresponding *P* value.

## Discussion

Low folate is causal in the pathology of NTDs in the human newborn and is associated with adult pathologies, including cancer, vascular disease, and dementias.[2−6] The potential benefit of increasing folate status across the population could be substantial. However, increasing folate intake by synthetic folic acid supplementation may in some circumstances be harmful.[18,25−27]

In this study we determined the effect of increasing synthetic folic acid intake on the human plasma proteome. Supplementing subjects with 1.2 mg of folic acid daily for 12 weeks significantly increased blood and intracellular folate status (Table 1). The changes described here were similar to those reported in the United States post folic acid fortification. Mean plasma folate (measured in the fifth and sixth waves of the Framingham Offspring Study birth cohort) rose from 4.6 to 10.0 μg/L, red cell folate increased from 325 to 450 μg/L, and total homocysteine concentrations fell from 10.1 to 9.4 μg/L postfortification.[7,8] Similar increases were reported from the NHANES cross-sectional population surveys (1999−2004), with serum folate doubling and red cell folate increasing more than 50% compared with prefortification levels.[28,29]

Folate supplementation induced significant changes in only a small number of human plasma proteins (apolipoprotein A1, α-1-antichymotrypsin, antithrombin III, serum albumin, serum amyloid P-component, IgM c chain, and complement C3),

**Table 5.** Correlation Analysis of Change in Folate Status and Human Plasma Proteins over the 12 Week Intervention[a]

| biomarker | spot | protein | correlation | *P* |
|---|---|---|---|---|
| plasma 5-methylTHF | 4002 | transthyretin | 0.588 | 0.0064 |
| plasma 5-methylTHF | 9504 | complement C3 | 0.567 | 0.0091 |
| red cell folate | 301 | α-2-HS-glycoprotein | 0.596 | 0.0056 |
| red cell folate | 8604 | complement factor B | 0.576 | 0.0079 |
| SAM | 1101 | complement C3 | 0.604 | 0.0092 |
| SAM | 5702 | complement factor H | 0.761 | 0.0006 |
| SAM | 7105 | IGKV1-5 protein | 0.621 | 0.0073 |
| uracil misincorporation | 1105 | clusterin | 0.710 | 0.0007 |
| uracil misincorporation | 1205 | clusterin | 0.674 | 0.0015 |
| uracil misincorporation | 1701 | complement C1s | 0.626 | 0.0039 |
| uracil misincorporation | 2106 | clusterin | 0.654 | 0.0023 |
| lymphocyte folate | 2302 | complement C4-B | −0.570 | 0.0087 |
| red cell folate | 5107 | apolipoprotein A-I | −0.567 | 0.0091 |
| SAM | 3202 | clusterin | −0.674 | 0.0031 |
| SAM | 3501 | α-1B-glycoprotein | −0.655 | 0.0043 |
| SAM | 6802 | α-2-macroglobulin | −0.893 | 0.0000 |
| uracil misincorporation | 2405 | kininogen-1 | −0.653 | 0.0023 |
| uracil misincorporation | 4101 | haptoglobin | −0.577 | 0.0091 |

[a] Blood biomarker, spot number, protein name, and correlation strength (positive and negative) are shown together with the corresponding *P* value.

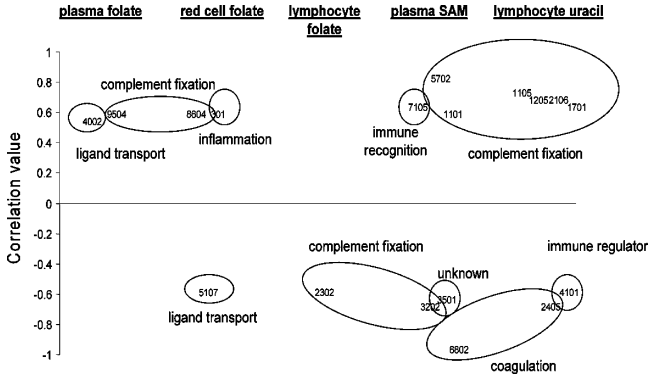


**Figure 1.** Strength of associations between change in folate status in response to intervention with synthetic folic acid and expression of plasma proteins involved in immune regulation and ligand transport. The spot number and protein function are grouped by blood biomarker for all subjects ($n = 20$). Protein identities and individual *P* values ($P < 0.01$ for all associations) are described in Table 5.

while correlation analysis revealed many plasma proteins, important in immune function, coagulation and fibrinolysis, regulation of blood pressure and lipid, and vitamin and mineral transport, both positively and negatively associated with blood and cellular folate, SAM, and homocysteine concentrations pre- and postintervention ($P < 0.01$). The implications of these changes are discussed below.

**Immune Function: Acute Phase Proteins and Complement Fixation.** Folate status, pre- and postintervention, was associated with expression of key acute phase proteins (e.g., transythretin, α-1-antichymotrypsin, α2-macroglobulin, ceruloplasmin, haptoglobin, hemopexin) and proteins involved in immune recognition (e.g., IgG-1 and IgM C chains, IGKV1-5, IgK light chain VLJ), complement activation (e.g., C1s, C4b, C3, factor B) and regulation (e.g., factor H, factor 1 (heavy chain), factor 1 light chain, clusterin, vitronectin). Complement, a biochemical cascade that removes pathogens from the body,[30] also mediates inflammation, adherence of immune cells, and phagocytosis of antigens by polymorphonuclear cells. Complement proteins account for 5−10% of the serum globin fraction. Three pathways interact in the complement system: (1) the classical pathway, (2) the alternative pathway, and (3) the mannose-binding lectin pathway. In the classical pathway, antigen is bound to IgG or IgM complexes with C1 (composed of subunits Cq, Cr, and Cs). Cs recruits C4 and C2 to form C3-convertase, which cleaves and activates C3 to C3a and C3b. C3a induces mast cell histamine release and vascular permeability. C3b splits C5 into C5a and C5b. C5a induces mast cell degranulation, lysosomal enzyme release, and vascular permeability. C5b complexes with C6 and C7 and recruits C8 and C9 to generate the "membrane attack complex" (MAC) that inserts itself into the cell membrane, forming a water- and electrolyte-permeable channel and inducing cell lysis. The alternative complement pathway bypasses requirement for antibody. Circulating C3 complexes with factor B to form C3bB. This is cleaved by factor D into Ba and Bb. Bb binds to C3b to form C3bBb, the alternative pathway C3 convertase, which acts on the same proteins (C5−C9) as in the classical pathway. Complement is tightly controlled to prevent harm to host cells. Factor 1 regulates complement activation by cleaving C3b and C4b. Factor H acts as a cofactor in factor 1 mediated C3b cleavage (as above) in both classical and alternative complement pathways and against C3 convertase (C3bBb). Vitronectin and clusterin prevent cytolysis by preventing insertion of the MAC into the plasma membrane.[30] A simplified diagram of the classical and alternative complement pathways is described in Figure 3, together with those proteins significantly associated with folate status (shown in red) in this study. Prior to intervention, folate status was significantly associated ($P < 0.01$) with the expression of several proteins functional in activation and regulation of both complement pathways (Table 3). Generally, high circulating folate was associated with high plasma concentrations of immunoglobulins and proteins related to complement activation and control. This positive relationship was maintained after supplementation with synthetic folic acid in the majority of the proteins examined with no net change in pathway function (Tables 2, 4, and 5). Several other acute phase proteins were linked with folate status. Hemopexin and haptoglobin prevent heme loss and heme-induced oxidative damage. Both are potent modulators of the immune response. Haptoglobin inhibits lymphocyte proliferation, granulocyte chemotaxis/phagocytosis, neutrophil respiratory burst activity,

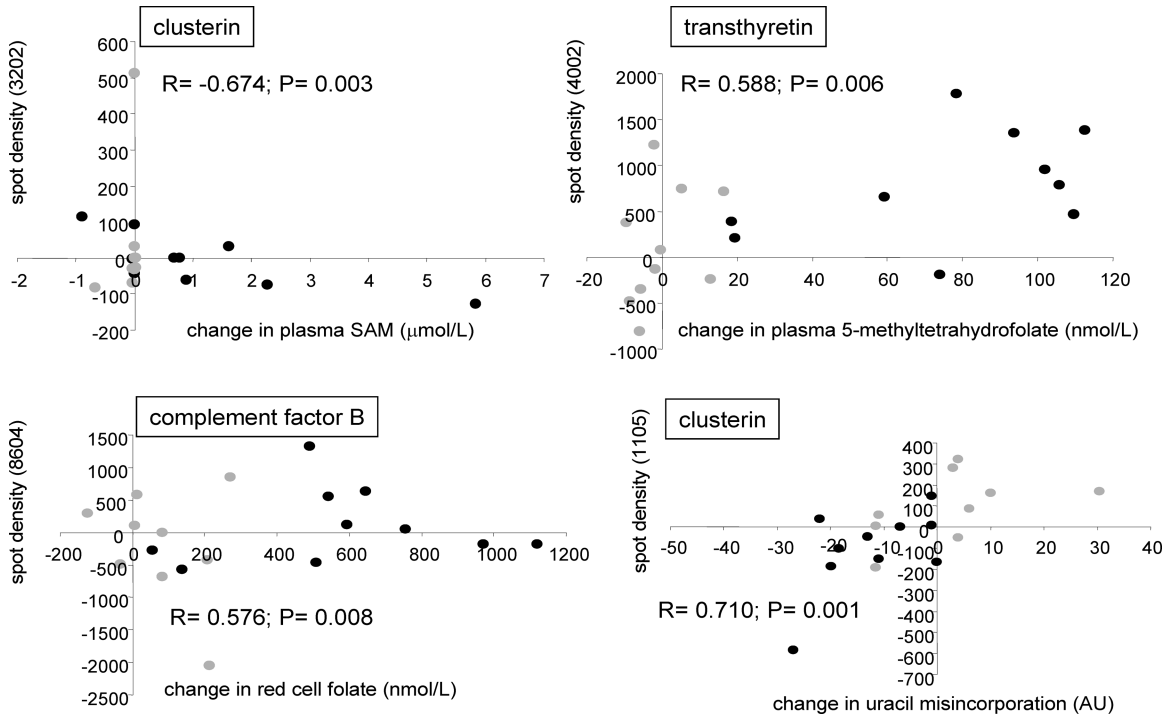


**Figure 2.** Scatter plots showing differential effects of increasing folate status on selected protein expression. Results are changes in blood folate status for all subjects (black circles denote folic acid intervention, $n = 10$; gray circles denote placebo, $n = 10$) and protein expression, together with strength of association (R) and corresponding probability ($P$) value. Sample ($n = 4$) proteins were chosen to highlight the associations between folate status and expression.

prostaglandin synthesis, and angiogenesis.[31−33] High folate status was associated with high haptoglobin expression pre- and postintervention. Hemopexin was positively associated with folate pre- and postintervention. α-1-Antichymotrypsin downregulates inflammation by inhibiting proteases and chymases.[34] α-1-Antichymotrypsin protein expression was significantly decreased (5-fold; $P < 0.05$) in plasma from supplemented subjects. α-2-HS-glycoprotein (fetuin-A), which regulates phagocytosis and inflammation,[35] was positively associated with red cell folate. α-2-Macroglobin protects cells from oxidative damage and regulates coagulation by inhibiting thrombin and plasmin.[36] Low α2-macroglobin expression was associated with high plasma SAM and low total homocysteine.

Diet has a profound influence on immunocompetence. Vitamins, minerals, trace elements, and amino and fatty acids are essential for effective immune function, while nutrient deficiencies in humans are associated with suppressed immunity and increased susceptibility to infection.[37,38] Micronutrients, including B vitamins, selenium, zinc, and iron, modulate both cellular and antibody mediated immune function,[37] preferentially supporting a proinflammatory cytokine response above an anti-inflammatory response.[38] Few studies have investigated how folate itself influences immunocompetence and whether folic acid (rather than folate) supplementation differentially modifies immunity. Feeding rats folic acid increases mitogen-stimulated lymphocyte proliferation and cytokine production in spleen and lymph tissue.[39] Folic acid supplementation reduces inflammatory cytokine MCP-1, IL-8, and CRP concentrations in obese human volunteers[40] and increases natural killer (NK) cell activity in women with a habitually low dietary folate intake.[41] However, the effects of increasing supplemental folic acid intake on immune function may not be universally beneficial. Consumption of synthetic folic acid (in excess of 400 μg/day) was associated with significantly lower NK cytotoxicity in women with a high dietary folate intake.[41] Moreover, plasma unmetabolized folic acid was associated with compromised NK cell activity.[41] Supplementing with megadoses of combined B vitamins (including folic acid) has been shown previously to impair human immune function.[42]

**Hemostasis and Coagulation.** Kiningen 1 (bradykinin) regulates blood pressure, diuresis, and coagulation.[38] Low kininogen expression was associated with low baseline SAM. Following supplementation, kininogen expression was positively associated with plasma total homocysteine and negatively with uracil misincorporation. Vitronectin (in addition to regulating complement) is involved in platelet adhesion and aggregation, coagulation, and fibrinolysis.[43] Low baseline vitronectin protein expression was associated with low folate status. Antithrombin III (antithrombin) inactivates enzymes of the coagulation pathway and serine proteases involved in complement.[34] Low baseline folate was associated with high antithrombin protein expression. Folate supplementation further decreased antithrombin expression (approximately 2-fold; $P < 0.05$). High folate status is therefore associated with low antithrombin expression and, presumably, high coagulation and complement activation potential.

**Ligand Binding and Transporter Proteins.** Proteins involved in vitamin, mineral, lipid, and hormone transport were strongly linked with blood folate status. Albumin, afamin, and vitamin D-binding protein, members of the albuminoid superfamily, act to regulate heme sequestering transport.[44] Albumin expression was consistently and positively associated with folate status before and after intervention. High afamin expression was associated with high homocysteine and low 5-methylTHF. High homocysteine was associated with low VDBP protein expression. Apolipoproteins maintain the structural integrity and solubility of lipoproteins for lipid

**Table 6.** Measures of Confidence for Protein Identification of Human Plasma Proteins Significantly Associated with Folate Status or Folic Acid Intervention[a]

| SSP | $M_r$(exptl) (kDa) | $M_r$(theor) (kDa) | pI | Mowse score, 10 log $P$ | no. of significant peptides matched | sequence coverage (%) | protein name | accession no. | gene name | function |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 12.01 | 23.39 | 4.36 | 301 | 5 | 28 | apolipoprotein A-I | P02647 | APOA1 | lipid metabolism and transport |
| 301 | 59.76 | 40.10 | 4.17 | 99 | 2 | 6 | α-2-HS-glycoprotein | P02765 | AHSG | bone mineralization; immune function |
| 402 | 64.1 | 49 | 4.29 | 412 | 7 | 15 | α-1-antichymotrypsin | P01011 | SERPINA3 | immune function |
| 1101 | 41.22 | 188.59 | 4.64 | 785 | 14 | 9 | complement C3 | P01024 | C3 | immune function |
| 1105 | 35.14 | 53.03 | 4.73 | 98 | 2 | 8 | clusterin | P10909 | CLU | immune function |
| 1203 | 41.27 | 188.59 | 4.58 | 263 | 6 | 5 | complement C3 | P01024 | C3 | immune function |
| 1205 | 37.62 | 37.00 | 4.68 | 97 | 2 | 7 | clusterin | P10909 | CLU | immune function |
| 1403 | 58.17 | 73 | 4.6 | 251 | 5 | 8 | kininogen-1 | P01042 | KNG1 | coagulation |
| 1503 | 79.99 | 55.13 | 4.71 | 235 | 5 | 8 | vitronectin | P04004 | VTN | immune function; coagulation |
| 1701 | 101.22 | 78.17 | 4.61 | 160 | 3 | 5 | complement C1s subcomponent | P09871 | C1S | immune function |
| 1702 | 101.7 | 106.2 | 4.56 | 53 | 2 | 4 | WD repeat-containing protein 22 | Q96JK2 | WDR22 | signal transduction |
| 2101 | 42.58 | 38.9 | 4.76 | 352 | 7 | 20 | haptoglobin | P00738 | HP | iron homeostasis; immune function |
| 2106 | 36.55 | 37 | 4.81 | 227 | 3 | 20 | clusterin | P10909 | CLU | immune function |
| 2302 | 46.7 | 40.80 | 4.86 | 137 | 2 | 6 | complement C4-B | P0C0L5 | C4B | immune function |
| 2405 | 58.15 | 48.9 | 4.89 | 172 | 4 | 8 | kininogen-1 | P01042−2 | KNG1 | coagulation |
| 3006 | 26.41 | 25.49 | 5.36 | 240 | 4 | 19 | serum amyloid P-component | P02743 | APCS | amyloid |
| 3101 | 38.3 | 28.2 | 5.1 | 144 | 3 | 12 | light chain of factor I | Q6LAM0 | CF1 | immune function |
| 3104 | 43.2 | 43.36 | 5.27 | 923 | 17 | 50 | apolipoprotein A-IV | P06727 | APOA4 | lipid metabolism and transport |
| 3202 | 35.42 | 37.00 | 5.13 | 143 | 3 | 18 | clusterin | P10909 | CLU | immune function |
| 3302 | 55.89 | 54.51 | 5.24 | 365 | 7 | 13 | vitamin D-binding protein variant | Q53F31 | GC | vitamin transporter |
| 3501 | 78.62 | 54.81 | 5.17 | 258 | 4 | 10 | α-1B-glycoprotein | P04217 | A1BG | unknown |
| 3502 | 77.51 | 54.7 | 5.27 | 318 | 6 | 14 | α-1B-glycoprotein | P04217 | A1BG | unknown |
| 3602 | 84.52 | 70.96 | 5.29 | 113 | 3 | 4 | afamin | P43652 | AFM | transport of unknown ligand |
| 4002 | 15.46 | 15.99 | 5.46 | 122 | 2 | 17 | transthyretin | P02766 | TTR | hormone and retinol transport |
| 4101 | 41.07 | 38.94 | 5.34 | 414 | 7 | 21 | haptoglobin | P00738 | HP | iron homeostasis; immune function |
| 4501 | 77.06 | 54.81 | 5.36 | 247 | 5 | 10 | α-1B-glycoprotein | P04217 | A1BG | unknown |
| 4604 | 143.86 | 123.43 | 5.48 | 227 | 4 | 5 | ceruloplasmin | P00450 | CP | iron homeostasis; immune function |
| 5107 | 23.9 | 23.39 | 5.65 | 122 | 3 | 15 | apolipoprotein A-I | P02647 | APOA1 | lipid metabolism and transport |
| 5202 | 43.22 | 68.13 | 5.71 | 164 | 4 | 7 | serum albumin | IBJ5 | ALB | multifunctional ligand transporter |
| 5301 | 54.95 | 53.03 | 5.53 | 369 | 7 | 20 | antithrombin-III | P01008 | SERPINC1 | coagulation |
| 5302 | 55.47 | 53.03 | 5.63 | 637 | 13 | 36 | antithrombin-III | P01008 | SERPINC1 | coagulation |
| 5501 | 73.03 | 67.69 | 5.5 | 268 | 5 | 13 | serum albumin | IAO6A | ALB | multifunctional ligand transporter |
| 5502 | 72.61 | 71.31 | 5.58 | 224 | 4 | 7 | serum albumin | ABHUS | ALB | multifunctional ligand transporter |
| 5601 | 143.4 | 98.32 | 5.57 | 279 | 6 | 11 | ceruloplasmin | P00450 | CP | iron homeostasis; immune function |
| 5702 | 186.35 | 143.71 | 5.64 | 456 | 10 | 10 | complement factor H | P08603 | CFH | immune function |
| 6105 | 24.09 | 23.39 | 5.83 | 83 | 2 | 10 | apolipoprotein A-I | P02647 | APOA1 | lipid metabolism and transport |
| 6301 | 54.51 | 47.66 | 5.72 | 163 | 3 | 8 | antithrombin III | P01008 | SERPINC1 | coagulation |
| 6501 | 71.13 | 52.39 | 5.74 | 186 | 5 | 10 | hemopexin | P02790 | HPX | iron homeostasis; immune function |
| 6503 | 69.88 | 52.39 | 5.91 | 366 | 5 | 12 | hemopexin | P02790 | HPX | iron homeostasis; immune function |
| 6802 | 129.69 | 164.60 | 5.84 | 296 | 5 | 3 | α-2-macroglobulin | P01023 | A2M | coagulation; immune function |
| 6804 | 128.2 | 164.60 | 5.96 | 500 | 9 | 7 | α-2-macroglobulin | P01023 | A2M | coagulation; immune function |
| 7001 | 19.05 | 38.94 | 6.12 | 191 | 3 | 4 | haptoglobin | P00738 | HP | iron homeostasis; immune function |
| 7102 | 27.27 | 25.49 | 5.98 | 149 | 2 | 2 | serum amyloid P-component | P02743 | APCS | amyloid |
| 7105 | 26.26 | 26.5 | 6.45 | 246 | 3 | 20 | IGKV1-5 protein | Q6PIH6 | IGKV1-5 | immune function |
| 7303 | 49.52 | 37.51 | 6.35 | 264 | 4 | 15 | heavy chain of factor I | Q6LAM1 | CF1 | immune function |
| 7405 | 60.84 | 71.45 | 6.48 | 130 | 2 | 5 | serum albumin | HSALB1 | ALB | multifunctional ligand transporter |
| 7601 | 95.08 | 67.69 | 6.32 | 138 | 2 | 4 | serum albumin | IAO6A | ALB | multifunctional ligand transporter |
| 7604 | 70.2 | 71.32 | 6.5 | 425 | 7 | 12 | serum albumin | ABHUS | ALB | multifunctional ligand transporter |
| 8105 | 24.77 | 67.69 | 6.5 | 106 | 3 | 6 | serum albumin | IAO6A | ALB | multifunctional ligand transporter |
| 8110 | 26.66 | 23.69 | 7.28 | 393 | 4 | 34 | IgG κ chain | P01834 | IGKC | immune function |
| 8303 | 48.15 | 71.18 | 6.74 | 267 | 4 | 9 | serum albumin | AAF01333 | ALB | multifunctional ligand transporter |
| 8304 | 48.46 | 67.69 | 6.59 | 434 | 9 | 14 | serum albumin | IA06A | ALB | multifunctional ligand transporter |
| 8404 | 69.33 | 71.34 | 6.58 | 810 | 13 | 20 | serum albumin | ABHUS | ALB | multifunctional ligand transporter |
| 8502 | 82.33 | 50.21 | 6.71 | 376 | 7 | 6 | IgM chain C region | P01871 | IGHM | immune function |

**Table 6.** Continued

| SSP | $M_r$(expt) (kDa) | $M_r$(theor) (kDa) | pI | Mowse score, 10 log $P$ | no. of significant peptides matched | sequence coverage (%) | protein name | accession no. | gene name | function |
|---|---|---|---|---|---|---|---|---|---|---|
| 8602 | 102.19 | 86.85 | 6.6 | 435 | 7 | 10 | complement factor B | P00751 | CFB | immune function |
| 8603 | 83.1 | 50.21 | 6.61 | 231 | 4 | 4 | IgM chain C region | P01871 | IGHM | immune function |
| 8604 | 101.61 | 86.85 | 6.69 | 465 | 8 | 12 | complement factor B | P00751 | CFB | immune function |
| 9402 | 50.43 | 36.6 | 6.88 | 210 | 4 | 16 | Ig $\gamma$-1 chain C region | P01857 | IGHG1 | immune function |
| 9504 | 74.17 | 188.57 | 7.31 | 248 | 5 | 3 | complement C3 | P01024 | C3 | immune function |
| 9605 | 75.02 | 188.59 | 7.18 | 341 | 5 | 5 | complement C3 | P01024 | C3 | immune function |
| 9702 | 75.34 | 188.57 | 7.02 | 429 | 9 | 7 | complement C3 | P01024 | C3 | immune function |

[a] Spot number, calculated and theoretical molecular weight (kDa), pI, accession number, and gene and protein names are presented for all identified proteins. Probability-based Mowse scores for LC MS/MS-based identifications, numbers of significant sequence peptides matched by "Mascot" searching (Matrix Science Ltd, Boston, MA), and percentage sequence coverages are shown.




**Figure 3.** Simplified diagram of the classical and alternative complement pathways. Those proteins identified in this study as being significantly associated with folate status are shown in red.

metabolism and transport.[45] Folic acid supplementation decreased expression of plasma Apo A-1, the major component of high-density lipoprotein (HDL), almost 9-fold ($P < 0.05$). Increasing red cell folate and lowering total plasma homocysteine were associated with low Apo A-1 levels postintervention. Preintervention, high circulating 5-methylTHF concentrations were associated with low Apo A-IV expression. These data indicate that low folate status is associated with high expression of Apo A-1 and A-IV and increasing folate status lowers protein expression. The impact this has on lipid metabolism and transport remains to be established.

This study reports the relationship between both dietary folate and supplemental synthetic folic acid intake on the plasma proteome. We observed that the expression of only a small number of proteins changed directly in response to folic acid intervention. This may reflect the relatively moderate dietary intervention.

Correlation analysis, exploring the associations between folate status and plasma protein expression both pre- and post-treatment, proved more useful. When many correlations are tested, the risk of false positives must be considered. The $q$ value of these analyses (mean $q = 0.65$ at $P = 0.01$ for change due to folate intervention) imply that it is likely that a proportion of spots found to be significantly influenced by folate status is due to chance. This is a common problem associated with proteomic analysis in plasma and blood cell samples from human nutritional trials, where interindividual variation in protein expression is high compared with data from in vitro or in vivo model studies.[23,24,46] Further investigation is required to determine which of the relationships reported here can be replicated. However, it is logical that those biochemical parameters that show greater numbers of correlations will have a lower false positive rate and so are individually more reliable. Moreover, correlations between change values must be interpreted carefully. Positive associations, for example, imply that more positive changes in one variable are associated with more positive changes in the other. This is different from stating that larger changes are associated if on average one variable increases and the other decreases. Nonetheless, even accounting for these caveats, in many cases the expression of a particular protein correlated with endogenous

folate levels and/or changes in folate status due to intervention measured in more than one biomarker and, more importantly, responded as would be expected biologically, giving a high degree of confidence to this type of analytical approach.

Here, we report that folate status is significantly associated with proteins primarily involved in immune function and that increasing folate intake using synthetic folic acid supplementation alters expression of these proteins, although homeostasis appears to be maintained. The data presented here indicate a "shift" rather than dramatic changes in pathways and do not provide strong evidence either in support of or against fortification with folic acid. Moreover, a clear mechanistic explanation for our observation that folate status significantly and differentially modulates numerous plasma protein expression related to immune function must be established. It remains to be confirmed whether folate status influences both expression of the genes regulating translation of these proteins and subsequent enzyme activity and what influence folate has on human cell mediated immune function.

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