Alanyl-Glutamine Consumption Modifies the Suppressive Effect of L-Asparaginase on Lymphocyte Populations in Mice

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Abstract

Asparaginase (Elspar) is used in the treatment of acute lymphoblastic leukemia. It depletes plasma asparagine and glutamine, killing leukemic lymphoblasts but also causing immunosuppression. The objective of this work was to assess whether supplementing the diet with glutamine modifies the effect of asparaginase on normal lymphocyte populations in the spleen, thymus, and bone marrow. Mice consuming water ad libitum with or without alanyl-glutamine dipeptide (AlaGln; 0.05 mol/L) were injected once daily with 0 or 3 international units/g body weight Escherichia coli L-asparaginase for 7 d. Tissue expression of specific immune cell surface markers was analyzed by flow cytometry. Asparaginase reduced B220+ and slgM+ cells in the bone marrow (P < 0.05) and diminished total cell numbers in thymus (−42%) and spleen (−53%) (P < 0.05). In thymus, asparaginase depleted double positive (CD4+CD8+) and single positive (CD4+CD8−, CD4−CD8+) thymocytes by over 40% (P < 0.05). In spleen, asparaginase reduced CD19+ B cells to 33% of controls and substantially depleted the CD4+ and CD8+ T cell populations. CD11b-expressing leukocytes were reduced by 50% (P < 0.05). Consumption of AlaGln did not lessen the effects of asparaginase in bone marrow or thymus but mitigated cellular losses in the CD4+, CD8+, and CD11b+ populations in spleen. AlaGln also blunted the increase in eukaryotic initiation factor 2 (eIF2) phosphorylation by asparaginase in spleen, whereas eIF2 phosphorylation did not change in thymus in response to asparaginase or AlaGln. In conclusion, asparaginase reduces maturing populations of normal B and T cells and differentially inhibits antibody-precursor cells in the bone marrow.

Introduction

Asparaginase is included in the induction regimen for treating acute lymphoblastic leukemia, the most common childhood cancer (1,2). Purified from bacteria, asparaginase is an enzyme that breaks down the amino acids asparagine and glutamine. The antileukemic properties of asparaginase are ascribed to the depleting of circulating asparaginase. Lymphoblastic tumor cells are thought to have a low ability to synthesize asparaginase [reviewed in (3)] and thus succumb to amino acid starvation-mediated cellular death pathways. Despite its effective ability to induce remission, many patients fail to successfully complete asparaginase treatment due to allergic reactions or cytotoxic complications such as liver dysfunction, pancreatitis, and immunosuppression (4–6). With respect to its recognized immunosuppressive properties, previous work has shown that in mice, asparaginase preferentially inhibits antibody-precursor cells in the bone marrow (7) and inhibits both cell-mediated and humoral immune responses (8). In mice, asparaginase inhibits immune responses against the T-dependent antigen, sheep red blood cells, and suppresses antibody responses, as reflected in decreased synthesis of antigen-specific IgG and IgM (9,10).

Previous work by our laboratory and others demonstrates that asparaginase depletes circulating and intracellular glutamine, inhibiting cellular growth and reducing protein synthesis at the level of mRNA translation initiation in spleen and other tissues or cell types (11–14). These responses were not present when mice were treated with a virtually glutaminase-free asparaginase isolated from the Wolinella (Vibrio) succinogenes microbe (12). Other studies have shown that both cell-mediated and humoral responses are not suppressed when mice are treated with this same glutaminase-free asparaginase (9,10). Glutamine is important in many cellular processes, notably for providing energy and nitrogen for the synthesis of DNA and RNA in lymphocytes, and serves to enhance the function of stimulated immune cells (15,16). Furthermore, glutamine is essential for optimal cell functioning of not only lymphocytes but also monocytes (17) and granulocytes (18). With respect to asparaginase, the depletion of glutamine is suggested to be the primary immunosuppressive agent (19).
Based on our previous work and the work of others, we hypothesized that the immunosuppressive effects of asparaginase are a result of decreased glutamine levels, leading to metabolic stress as evidenced by an increase in the phosphorylation of the translation factor, eukaryotic initiation factor 2 (eIF2)\(^5\). We endeavored to prevent or ameliorate this condition by increasing the supply of glutamine in the diet via unlimited consumption of an alanyl-glutamine dipeptide (AlaGln) solution. The effect of asparaginase alone and in combination with AlaGln consumption on the major lymphocyte subpopulations in bone marrow, spleen, and thymus of normal mice was explored by determining the expression of various lymphocyte cell surface markers by flow cytometry.

**Materials and Methods**

**Animals and experimental design.** The experimental protocol was approved by the Institutional Animal Care and Use Committee of the Indiana University School of Medicine-Evanville campus. During each experiment, male and female C57BL/6j mice (6–8 wk old) were housed in wire-bottomed cages, maintained on a 12:12-h light:dark cycle and provided free access to a standard commercial rodent food based on AIN-93 standards (20) consisting of 18% protein and 4% fat (7017 NIH-31 Open Formula Mouse/Rat Sterilizable diet, Harlan Teklad). Mice were acclimated to the wire-bottom format for at least 3 d prior to the experimental protocol. AlaGln was used as the source of dietary glutamine, because it is highly stable in solution and is reported to increase plasma glutamine in mammals when ingested (21–23). On d 1 of the experiment, mice (6 per group) were provided free access to a drinking solution of either water or 0.05 mol/L AlaGln. Mice were acclimated to the wire-bottom format for at least 3 d prior to the experimental protocol. AlaGln was used as the source of dietary glutamine, because it is highly stable in solution and is reported to increase plasma glutamine in mammals when ingested (21–23). On d 1 of the experiment, mice (6 per group) were provided free access to a drinking solution of either water or 0.05 mol/L AlaGln. Body weight, food intake, and fluid intake were measured daily. On d 2–7, mice received once-daily intraperitoneal injections of PBS or PBS containing an enzyme activity of 3 international units (IU) of *Escherichia coli* l-asparaginase per gram body weight. Treatment groups were designated as follows: PBS-injected mice with no AlaGln in drinking water (WS); PBS-injected mice provided AlaGln in drinking water (GS); asparaginase-injected mice provided pure water (WA); asparaginase-injected mice provided AlaGln in drinking water (GA). The dose of asparaginase chosen was based on our published work (12) and a previous report that states that mice are resistant to the toxicity of asparaginase up to 2000 IU/kg body weight (24). The number of doses (6 daily injections) is based on the works of Durden et al. (9,10,25,26), which demonstrated that 4–7 d of asparaginase treatment resulted in maximal cytotoxicity and immunosuppression. Durden et al. (9,10,25,26), which demonstrated that 4–7 d of asparaginase treatment resulted in maximal cytotoxicity and immunosuppression. Daily injections were given because the half-life of *E. coli* asparaginase in mice is ~3 h, whereas in humans, it is on the order of 30 h (27). The dose of AlaGln was decided following a pilot study examining several concentrations of the dipeptide, ranging from 0.02–0.5 mol/L. This pilot study indicated that the 0.05 mol/L concentration was the highest that would not influence food intake, fluid intake, or somatic growth (our unpublished data). All mice were killed on d 7 by decapitation 3 h after the last injection. Trunk blood was collected and allowed to clot on ice to obtain serum following centrifugation. Tissues were rapidly harvested and organ weights were measured.

**Measurement of l-asparaginase activity.** The activity of experimental l-asparaginase derived from *E. coli* (Elspar product from Merck & Co.) was determined prior to administration by the Nesslerization technique, as described previously (12,28,29).

**Cellular composition of bone marrow, spleens, and thymi.** The major component cell types within spleens, thymi, and bone marrow were determined using flow cytometric analysis protocols as described (30). Fluorescein isothiocyanate (FITC)- or phycoerythrin (PE)-labeled antibodies against cell surface markers were purchased from BD Biosciences unless otherwise noted. Briefly, splenocytes, thymocytes, or bone marrow cells (0.5 × 10⁶ cells/sample) were washed once in fluorescence-activated cell sorting (FACS) buffer (PBS with 1% fetal bovine serum and 0.05% sodium azide) and incubated on ice in the presence of specific monoclonal antibodies for ~20 min in the dark. The cells were washed twice, fixed in 1% parafomaldehyde, and cell surface marker expression detected on a FACSscan flow cytometer (Becton Dickinson). Data were analyzed using CellQuest Pro software (Becton Dickinson), gating on 10,000 lymphocytes as defined by forward and side scatter. Markers of T cell development and function included CD3 (FITC-17A2), CD4 (PE-GL1.5), and CD8 (FITC-53-172). B cell markers included CD19 (FITC-1D3), surface IgM (FITC-µ-chain, MP Biomedicals), and B220 (PE-RA3–6B2). CD11b (PE-Mac-1 chain) was examined as a marker of leukocytes in spleen. Isotype-matched antibodies against IgG2a, B39–4), IgG2b, (A95–1), and IgM (R4–22) were used as controls. The percent positive cells for each subpopulation of the thymus and spleen were adjusted based on total cell numbers in that tissue and data are presented as 10⁶ cells per organ. Bone marrow data are presented as percent positive cells for each subpopulation. Total cell count of each organ was determined using a Coulter Counter.

**Serum amino acids.** Serum was obtained by centrifugation of clotted blood and stored at ~20°C. Serum samples were sent to the Indiana University School of Medicine Quantitative Amino Acid Core Facility (Director Edward Liechty, M.D.) for the determination of amino acid profiles by the ninhydrin method, using standard ion exchange chromatography with a Beckman 6300 automated amino acid analyzer.

**Immunoblot analysis.** Phosphorylation of eIF2 was assessed as previously described (12) using an antibody that recognizes the α-subunit only when it is phosphorylated at Ser-51 (Cell Signaling Technology). Results were normalized for total eIF2a with an antibody that recognizes the protein irrespective of phosphorylation state (Santa Cruz Biotechnology). Phosphorylation of eIF4E binding protein-1 (4E-BP1) and ribosomal protein S6 kinase (S6K1) were measured by protein immunoblot analysis as described previously (12) (Bethyl).

**Statistics.** All data were analyzed by the STATISTICA statistical software package for Macintosh, volume II (StatSoft). Data were analyzed using 2-way ANOVA to assess main vs. interaction effects, with asparaginase treatment and AlaGln as independent variables. When variances among treatment groups were unequal, the data were log transformed before statistical analysis to achieve homogeneity of variances. Differences between individual treatment groups were assessed using Tukey’s honestly significant difference (HSD) post hoc test for unequal sample size. The data presented are expressed as means ± SEM, except Table 1, which displays means ± SD. The level of significance was set at \( P < 0.05 \) for all statistical tests.

**Results**

**Food intake, fluid intake, AlaGln intake, and body weight gain.** Mean daily energy and fluid intakes of WS controls were 67.7 ± 0.3 kcal/d and 6.2 ± 0.3 mL/d, respectively. Neither asparaginase nor dietary glutamine altered food or fluid intake (data not shown). Even after accounting for the additional energy provided by AlaGln consumption (88.3 ± 5.2 mg/d AlaGln), total energy consumed was similar among treatment groups. Initial body weights did not differ among the WS (18.0 ± 1.3 g), GS (20.5 ± 1.0 g), WA (19.6 ± 1.4 g), and GA (20.4 ± 1.0 g) groups. Body weight gain over 7 d was inhibited by asparaginase; it was 1.02 ± 0.24 g in saline-injected mice (WS and GS) and ~0.012 ± 0.078 g in WA mice (\( P < 0.05 \)). Body weight gain in GA mice (0.55 ± 0.23 g) was intermediate and not significantly different from other groups.

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\(^5\) Abbreviations used: AlaGln, alanyl-glutamine dipeptide; eIF2, eukaryotic initiation factor 2; 4E-BP1, eIF4E binding protein-1; FACS, fluorescence-activated cell sorting; FITC, fluorescein isothiocyanate; GA, mice consuming glutamine dipeptide in the tap water alongside receiving injections of asparaginase; GS, mice consuming glutamine dipeptide in the tap water alongside receiving injections of PBS; HSD, honestly significant difference; IU, international unit; PE, phycoerythrin; WA, mice consuming tap water alongside receiving injections of asparaginase; WS, mice consuming tap water alongside receiving injections of PBS.
**Table 1** Serum amino acid concentrations in mice injected once daily with L-asparaginase for 7 d with or without oral AlaGln supplementation.

<table>
<thead>
<tr>
<th>µmol/L</th>
<th>WS</th>
<th>GS</th>
<th>WA</th>
<th>GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine*</td>
<td>415.8 ± 175.5</td>
<td>270.4 ± 29.0</td>
<td>377.3 ± 40.2</td>
<td>547.5 ± 120.4</td>
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<tr>
<td>Arginine*</td>
<td>161.3 ± 38.1</td>
<td>113.2 ± 30.2</td>
<td>172.2 ± 41.7</td>
<td>202.2 ± 56.6</td>
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<tr>
<td>Aspartic acid*</td>
<td>9.1 ± 5.5</td>
<td>13.6 ± 4.6</td>
<td>21.3 ± 5.4</td>
<td>19.7 ± 4.6</td>
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<tr>
<td>Asparagine*</td>
<td>35.2 ± 8.8</td>
<td>26.7 ± 7.3</td>
<td>&lt;1</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Glutamic acid*</td>
<td>87.4 ± 25.8</td>
<td>98.9 ± 3.8</td>
<td>40.1 ± 122.8</td>
<td>45.8 ± 66.7</td>
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<tr>
<td>Glutamine</td>
<td>607.1 ± 70.2</td>
<td>701.1 ± 28.2</td>
<td>595.7 ± 32.0</td>
<td>622.2 ± 85.6</td>
</tr>
<tr>
<td>Glycine*</td>
<td>206.8 ± 27.2</td>
<td>228.3 ± 18.9</td>
<td>344.7 ± 52.7</td>
<td>405.4 ± 50.0</td>
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<tr>
<td>Histidine</td>
<td>69.7 ± 26.8</td>
<td>70.6 ± 14.5</td>
<td>68.2 ± 13.3</td>
<td>76.2 ± 8.6</td>
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<tr>
<td>Isoleucine</td>
<td>102.7 ± 26.3</td>
<td>56.4 ± 7.8</td>
<td>79.9 ± 17.8</td>
<td>110.7 ± 36.1</td>
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<tr>
<td>Leucine</td>
<td>176.9 ± 39.7</td>
<td>105.1 ± 20.7</td>
<td>144.2 ± 31.6</td>
<td>202.2 ± 61.7</td>
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<tr>
<td>Lysine</td>
<td>383.4 ± 129.1</td>
<td>266.1 ± 34.5</td>
<td>396.4 ± 172</td>
<td>487.5 ± 194.6</td>
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<tr>
<td>Methionine</td>
<td>76.3 ± 41.3</td>
<td>41.7 ± 4.3</td>
<td>56.9 ± 6.8</td>
<td>80.4 ± 24.7</td>
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<tr>
<td>Proline</td>
<td>97.7 ± 44.1</td>
<td>62.5 ± 8.7</td>
<td>90.7 ± 16.9</td>
<td>110.1 ± 30.4</td>
</tr>
<tr>
<td>Serine*</td>
<td>99.2 ± 16.4</td>
<td>101.2 ± 20.9</td>
<td>142.4 ± 24.8</td>
<td>173.3 ± 46.7</td>
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<tr>
<td>Threonine</td>
<td>143.4 ± 60.4</td>
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<td>Valine</td>
<td>233.2 ± 52.7</td>
<td>144.4 ± 12.2</td>
<td>193.3 ± 29.1</td>
<td>267.2 ± 82.4</td>
</tr>
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</table>

1 Values are means ± SD, n = 4–6. When necessary, data were transformed using the natural log of each value before statistical analysis to achieve homogeneity of variance. *Main effect of asparaginase, P < 0.05; †main effect of AlaGln, P < 0.05. When a significant asparaginase × AlaGln interaction was present, Tukey’s unequal sample size HSD post hoc test was used to reveal differences among treatment groups, P < 0.05. ‡Different from WS; ‡different from GS.

**Circulating amino acid concentrations.** Asparaginase treatment severely reduced circulating asparagine concentrations below the instrument detection limits (<1 µmol/L) and aspartic acid concentrations more than double in the serum of mice treated with asparaginase (P < 0.05) (Table 1). On the other hand, AlaGln consumption increased serum glutamine and glycine concentrations (P < 0.05). Asparaginase alone did not alter serum glutamine, but all asparaginase-treated mice had increased circulating glycine acid, as well as serum alanine, arginine, glycine, and serine (P < 0.05). Serum alanine and arginine were highest in GA mice and serum concentrations of the BCAA were reduced in GS mice compared with WS controls (interaction, P < 0.05). All other amino acids measured did not differ from those of the WS group in mice treated with asparaginase and/or AlaGln.

**Immune cell populations in bone marrow.** Relative to WS controls, asparaginase reduced the proportion of bone marrow cells expressing B220 (B220⁺ B cells) or surface IgM (slgM⁺ B cells) compared with mice injected with saline (P < 0.05) (Fig. 1). Thus, the bone marrow in WA mice was depleted of B220⁺slgM⁻ and B220⁺slgM⁺ B lymphocytes, shifting the balance of the gated population toward an increased proportion of B220⁻slgM⁻ cells (P < 0.05). AlaGln did not alter the distribution of cells expressing B220⁺ or slgM⁺ and did not mitigate the proportional loss in maturing B cells by asparaginase. The groups did not differ in the proportion of bone marrow cells expressing the hematopoietic stem cell marker, CD117/c-kit (data not shown).

**Immune cell subpopulations in thymus.** Asparaginase decreased thymus weight, total thymocyte numbers, and CD3⁺ thymocytes (P < 0.05) (Fig. 2A,B). Cell loss was not detected in the most immature (CD4⁻CD8⁻) population of thymocytes (Fig. 2C). Instead, asparaginase substantially depleted both double positive (CD4⁺CD8⁺) and single positive (CD4⁺CD8⁻, CD4⁻CD8⁻) thymocytes (P < 0.05) (Fig. 2D–F). AlaGln did not reverse the thymocyte population losses due to asparaginase treatment.

**Immune cell populations in spleen.** Asparaginase decreased spleen wet weight and total cell number (P < 0.05) (Fig. 3A,B). Asparaginase treatment also reduced the proportion of cells expressing CD4⁺ or CD8⁺ (P < 0.05) (Fig. 3C,D). Furthermore, asparaginase decreased CD19⁺ B cells and CD11b⁺ leukocytes (P < 0.05) (Fig. 3E,F). Supplementing the diet with AlaGln increased the proportion of splenocytes expressing CD4⁺ and CD8⁺ (P < 0.05). Although AlaGln did not prevent loss of CD19⁺ B cells, the loss of CD11b⁺ cells in response to asparaginase was partially prevented (interaction, P = 0.069).

**Phosphorylation status of translation factors.** Asparaginase increased the phosphorylation of eIF2 in spleen (P < 0.05) (Fig. 4). AlaGln slightly blunted eIF2 phosphorylation in spleen (interaction, P < 0.05) but did not alter the phosphorylation state of eIF2 in thymus either alone or in combination with asparaginase (data not shown). Phosphorylation states of 4E-BP1 and ribosomal protein S6 kinase 1 in spleen or thymus did not differ, with all treatment groups similar to WS controls (data not shown).

**Discussion**

In this study, we confirmed that asparaginase effectively depletes immune cell populations in both the primary and peripheral lymphoid systems. Lymphocytes in the bone marrow, thymus, and spleen of healthy mice were reduced substantially following 6 daily injections of asparaginase. Importantly, oral consumption of AlaGln lessened eIF2 phosphorylation and prevented cellular losses of CD4⁺ and CD8⁺ T lymphocytes and leukocytes expressing the integrin, CD11b, in spleen. These data suggest that dietary supplementation of glutamine may benefit cell-mediated immunity during asparaginase chemotherapy.

Following 6 d of treatment, asparaginase continued to effectively exhaust circulating asparagine, whereas circulating glutamine was maintained at normal concentrations. Asparaginase is
recognized to thwart the production of asparagine in part by breaking down glutamine, a necessary substrate in the asparagine synthetase reaction (aspartate + glutamine + ATP → asparagine + glutamate + AMP + pyrophosphate). Clinically, asparaginase reduces fasting plasma glutamine in pediatric patients with acute lymphoblastic leukemia (11) and glutamine deamination activity is correlated with serum asparaginase activity (31). Furthermore, we reported a single injection of *E. coli* asparaginase lowers both plasma and intracellular glutamine in healthy mice (12). Taken together, our previous work and the current data indicate that circulating concentrations of glutamine initially fall, but then rebound over time. This may be due to an enhanced production of glutamine, with subsequent efflux into the circulation to support other tissues with a lower capacity to make glutamine.

In the bone marrow, asparaginase reduced cell populations expressing B220<sup>+</sup> or sIgM<sup>+</sup>, reflecting predominantly maturing B cells. In cancer patients, the most asparaginase-sensitive populations in bone marrow are of the B cell lineage (7). This study confirms that asparaginase reduces a substantial proportion of bone marrow-derived normal lymphocytes, particularly those involved in the humoral immune response. Interestingly, despite drastic reductions in the B220<sup>+</sup> and sIgM<sup>+</sup> populations, asparaginase did not alter the population of cells expressing c-kit/CD117<sup>+</sup>. This suggests that normal hematopoietic stem cells and multi-lineage progenitors, which in the bone marrow are delineated by the expression of this cell-surface molecule (32), are relatively insensitive to asparaginase. It is unknown if c-kit/CD117<sup>+</sup> cells possess more asparagine synthetase or glutamine synthetase activity compared with a more differentiated lymphocyte. Alternatively, a recent study reports that asparagine synthetase expression in bone marrow-derived mesenchymal cells is much higher than in leukemic lymphoblasts and in coculture experiments protects leukemic cells from asparaginase cytotoxicity (33). Perhaps these cells could also protect hematopoietic multi-lineage progenitors from asparagine and/or glutamine depletion. Further study is required to more specifically delineate the cellular and environmental factors that influence the normal lymphoid cell response to asparaginase.

In this study, asparaginase did not alter the double negative population but instead depleted the CD4<sup>-</sup>CD8<sup>-</sup> double positive...
subpopulation. These data suggest that asparaginase interferes with the events necessary for simultaneous surface expression of both CD4 and CD8 proteins. Why thymocytes are sensitive to asparaginase at this intermediate stage of development requires further study but may be connected with the increased burden of new proteins to synthesize to survive positive and negative selection.

Although the flow cytometric data clearly shows that primary and peripheral lymphocyte populations decline following asparaginase, how they are lost is not revealed. Previous studies have shown that treatment of leukemic cells with E. coli asparaginase causes the fragmentation of chromosomal DNA and arrest in the G1 phase (34,35). Data from our laboratory demonstrate an increase in phosphorylated eIF2 (Fig. 4), induction of the pro-apoptotic transcription factor, CHOP (12), and caspase-3 cleavage in the spleen 6 h after a single injection of asparaginase (our unpublished data). Thus, loss of lymphocytes is in part a result of increased cell stress leading to cell death via apoptosis. However, asparaginase may also cause necrosis in addition to programmed cell death. Certainly, this type of cell damage is reported in the pancreas, liver, bone, and bone marrow of patients receiving asparaginase (36–39). Further study into the factors that determine whether the cells in each tissue undergo apoptosis vs. necrosis when exposed to asparaginase is needed.

Although supplementation of dietary glutamine did not prevent cellular depletion by asparaginase in bone marrow or thymus, it was partially successful at mitigating losses in CD4+ and CD8+ T cells and cells expressing CD11b/Mac-1 in the spleen. CD11b/Mac-1 is a member of the β2-integrin family of adhesion molecules (40). It is expressed on monocytes, neutrophils, peritoneal B-1 (CD5) cells, CD8+ dendritic cells, NK cells, and a subset of CD8+ T cells and functions as a heterodimer associated with the common β2 chain (CD18) (41). CD11b plays important roles in cellular adhesion, phagocytosis and extravasation, and chemotaxis and neutrophil respiratory burst (40). It also binds a diverse group of ligands, which includes iC3b, fibrinogen, coagulant factor X, and the intercellular adhesion molecule ICAM-1 (42). A previous study indicated that high CD11b+ expression defines a subset of circulating effector CD8+ T cells that were recently activated (43). Taken together, the current results in combination with the literature suggest that dietary AlaGln supplementation may benefit the functioning of peripheral effector T cells and/or promote leukocyte migration or adhesion. This important finding supports further evaluation of AlaGln supplementation to support innate as well as T cell-mediated immunity in patients treated with asparaginase.

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