Supplementation with Active Hexose Correlated Compound Increases the Innate Immune Response of Young Mice to Primary Influenza Infection

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Abstract

The emergence of H5N1 avian influenza and the threat of new or adapted viruses in bioterrorism have created an urgent interest in identifying agents to enhance the immune response to primary virus infection. Active hexose correlated compound (AHCC) is a natural mushroom extract reported to increase natural killer (NK) cell activity, survival, and bacterial clearance in young mice. However, the effects of AHCC on the response to viral infections have not been studied. In this study, young C57BL/6 mice were supplemented with 1 g AHCC/(kg body weight) for 1 wk prior to and throughout infection with influenza A (H1N1, PR8). Supplementation increased survival, decreased the severity of infection, and shortened recovery time following intranasal infection with flu, as determined by the recovery of body weight and epithelial integrity in the lungs. AHCC increased NK activity in lungs at d 1 ($P < 0.05$) and d 4 ($P < 0.01$) and in the spleen at d 2 postinfection ($P < 0.01$). Supplementation increased the percentage ($P < 0.05$) and number ($P < 0.01$) of NK1.1+ cells in the lung and reduced the infiltration of lymphocytes and macrophages compared with controls ($P < 0.01$). These data suggest that AHCC supplementation boosts NK activity, improves survival, and reduces the severity of influenza infection in young mice. Bolstering innate immunity with dietary bioactives may be one avenue for improving the immune response to primary flu infection. J. Nutr. 136: 2868–2873, 2006.

Introduction

Influenza virus is a public health concern in the United States, causing disease among all age groups. Although children demonstrate the highest rates of infection, rates of serious illness and death are highest among persons aged ≥65 y, young children aged <2 y, and persons of any age with medical conditions putting them at increased risk for complications from influenza. Among children aged 0–4 y, hospitalization rates have ranged from ~1 in 1000 for children without high-risk medical conditions to 1 in 200 for those with high-risk medical conditions (1). Within the 0–4 y age group, hospitalization rates are highest among children aged 0–1 y and are comparable to rates reported among persons aged ≥65 y (1). Thus, in 2004–05, the Advisory Committee on Immunization Practices (ACIP) recommended that all children aged 6–23 mo receive yearly influenza vaccinations (1).

The highest rates of infection with influenza virus occur in young children, in part because of a lack of prior immunity from previous exposure to the virus. Immune immune systems rely heavily on innate defenses. Natural killer (NK) cells require neither prior exposure to virus nor antigen presentation to target and kill virus infected cells and, thus, provide one of the first lines of defense against many different viral infections, including influenza. NK cell–mediated killing controls viral replication until the virus is cleared by the adaptive immune response. However, in some cases, a sufficient NK cell response may eliminate an infection completely (2). The roles of NK cells in controlling influenza infection at the site of infection, i.e., the lung, and in activating adaptive, antigen-specific immunity in response to primary influenza infection have not been fully characterized (3–5). We (6,7) and others (8,9) have found an increase in NK activity in the lungs of young mice following influenza infection. Young mice subjected to restraint stress and infected with intranasal (i.n.) influenza virus demonstrated suppressed NK cell activation and function that was followed by enhanced viral replication (8). Depletion of pulmonary NK cells increases the mortality of mice infected with influenza and delays the initiation of a virus-specific CD8+ T cell response (5). In our previous studies, a reduction in NK response to influenza infection

1 This work was funded in part by a nonrestrictive grant from Amino Up Chemical Co., Sapporo, Japan, the manufacturer of the agent that was studied.

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2 Abbreviations used: AHCC, active hexose correlated compound; CTL, cytotoxic T lymphocyte; FBS, fetal bovine serum; HAU, hemagglutination units; H & E, hematoxylin-eosin Y; IHC, immunohistochemistry; i.n., intranasal; NK, natural killer; TCID, tissue culture infective dose.

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infection in aged energy-restricted mice was associated with an increase in viral titers in lungs and early mortality at day 4 post-infection, before the initiation of a CTL response could be generated (7). Although basal NK activity does not differ between young and aged mice, there is an age-associated decline in cytokine-inducible NK activity that is associated with a delay in viral clearance and a decreased and delayed adaptive response (3, 5, 6). A decrease in inducible NK activity has also been observed in aging humans (4). These data clearly indicate that NK cells are important in maintaining both the innate and adaptive immune responses and in controlling virus burden during primary influenza infection. Efforts to enhance the activation of NK cells involved in innate immunity, therefore, would also be expected to lead to the subsequent enhancement of adaptive immune responses. As a result, inducible NK activity is a potential therapeutic target of current interest (9).

In this study, we examined the effect of a dietary supplement known as active hexose correlated compound (AHCC) on the influenza-induced NK cell response during primary influenza infection in young mice. This compound is an enzyme-fermented extract of the mycelia of Basidiomycetes mushrooms and is marketed in the U.S. as a dietary supplement, or nutraceutical, containing a mixture of polysaccharides, amino acids, lipids, and minerals. The predominant components of AHCC are oligosaccharides, totaling ~74% of the mixture. Of these, nearly 20% are partially acetylated α-1,4-glucans with an mean molecular weight of 5000. These oligosaccharides are believed to account for the biological activities of AHCC. Supplementation with AHCC has shown a generalized positive effect on the immune systems of both rodents (10–14) and humans (15, 16), as well as antioxidant effects (17, 18), and is well-tolerated by both immune systems of both rodents (10–14) and humans (15, 16), as well as antioxidant effects (17, 18), and is well-tolerated by both rodents and humans, with no reported adverse effects. Studies to date have suggested that AHCC may increase NK activity in humans (16) and rodents (11, 14) with malignancies. In response to infection, AHCC supplementation increased percent survival, mean time until death, and bacterial clearance (Klebsiella pneumoniae) in young mice stressed by 15–20% head-down tilt (10). However, no studies have previously examined the effect of AHCC supplementation on the immune response to influenza infection or viral clearance.

Methods

Animals. The protocol was approved by the Drexel University Institutional Animal Care and Use Committee. Specific pathogen-free young (6- to 8-wk-old) male C57BL/6 mice were obtained from Jackson Laboratories. Mice were housed in microisolation cages in a barrier room of the AAALAC-accredited animal facility at Drexel University and acclimated for at least 1 wk before the initiation of experiments. Mice were monitored and weighed daily.

Supplementation with AHCC. AHCC (Amino Up Chemical Company) was administered orally by pipette at a concentration of 1 g AHCC/kg of body weight · d in 20 μL distilled water. Control mice received 20 μL distilled water per d. Mice were supplemented for 7 d prior to and throughout the course of infection with influenza. This dose of AHCC has been used previously and does not produce toxic effects in young mice (10).

Virus. Influenza A/Puerto Rico/8/34 (PR8, H1N1; a kind gift from Dr. Walter Gerhardt, University of Pennsylvania) was propagated in specific pathogen-free eggs (B & E Eggs) and cell-free supernatants were stored at –70°C for subsequent use. At baseline (d 0), mice were anesthetized by intraperitoneal injection with Avertin (2,2,2-tribromoethanol, Sigma) and were infected i.n. with 100 hemagglutination units (HAU) of PR8.

Isolation of mononuclear cells from spleens and lungs. The procedure for the isolation of mononuclear cells from spleens and lungs has been described in detail previously (19). Briefly, mice were killed by CO2 asphyxiation, and spleens and lungs were aseptically removed. Spleens were homogenized by dounce and resuspended in RPMI-1640 (Mediatech). Lungs were minced with dissecting scissors and incubated at 37°C for 1.5 h in a cocktail containing 3 mg/mL Collagenase A and 80 Kunitz units of DNASE l/mL (Roche) with 5% fetal bovine serum ([FBS] Mediatech) in Iscove’s Modified Dulbecco’s Medium ([IMDM] Mediatech). The digested lung samples were passed through a 40-μm nylon mesh (Fisher) and centrifuged (500 × g; 5 min). Supernatants were aliquoted and stored at –70°C for subsequent analysis of virus titers. The pellets were resuspended and washed twice with 5% FBS in IMDM. The cell suspensions from spleens and lungs were layered on Histopaque-1083 (Sigma) and subjected to density gradient centrifugation (1400 × g; 20 min). Cells from each tissue were resuspended to the appropriate concentration for use in subsequent assays.

Lung virus titers. Supernatants from lung homogenates were serially diluted and used to infect Madin-Darby canine kidney cells. After incubation at 37°C for 24 h, 0,02% TPCK-treated trypsin (Sigma) was added, followed by an additional 48-h incubation. Chicken red blood cells (B & E Eggs) were resuspended at 0.05% in PBS and added to the cultures. Virus titers were then determined, based on the presence or absence of hemagglutination as previously described (19), and reported as the 50% tissue culture infectious dose (TCID50).

NK cell activity in lungs. We used a standard 4-h 51Cr-release assay with YAC-1 target cells to assess NK activity as previously described (6). Briefly, 1 × 105 YAC-1 cells were incubated with 200 μCi Na51CrO4 (PerkinElmer) for 2 h at 37°C. During this incubation, cells were mixed every 20 min to ensure maximal uptake of Na51CrO4. The cells were then washed twice with RPMI-1640, resuspended in RPMI-1640 complete medium containing 10% FBS, and rotated for 1 h at room temperature. After the final wash, YAC-1 cells were resuspended at 1 × 106 cells/L in complete medium and plated in flat-bottom 96-well microtiter plates (VWR). Cell preparations were then added to wells at an effector to target (E:T) ratio of 50:1. All samples were assayed in triplicate. Target cells were incubated in medium alone to assess spontaneous release or with 5% Triton X-100 (Sigma) to quantitate maximum release. After a 4-h incubation at 37°C, supernatants were harvested onto UniFilter microplates (PerkinElmer), and radioactivity in supernatants was quantitated using a γ-counter (Packard TopCount). Percent cytotoxicity was calculated as follows: % Cytotoxicity = (Experimental CPM − Spontaneous CPM) / (Maximum CPM − Spontaneous CPM) × 100. Spontaneous release was always <5% of maximal release.

Immunophenotyping. Following multiple washes, 5 × 105 mononuclear cells from spleens or lungs were resuspended in PBS containing fluoresochrome-conjugated antibodies (eBioscience) to CD4 (Pe-Cy7), CD8 (APC), NK1.1 (PE), and CD11b (FITC) and incubated on ice in the dark for 30 min. Cells were then washed 3 times in HBSS (Mediatech) containing 1% FBS, resuspended in PBS containing 1% paraformaldehyde (Sigma), and stored at 4°C until analysis. Samples were acquired on a FACScanto flow cytometer (Becton Dickinson) and analyzed using FACSdiva software.

Tissue staining. Formalin-fixed lung tissue was paraffin embedded, sliced, and mounted onto glass slides. Slides were baked at 65°C for 30 min and deparaffinized by xylene wash. Rehydration of tissue was carried out through a graded alcohol series (100%, 95%, and 80%). Slides were then washed and either stained for histology using the hematoxylin-eosin Y (H & E) method or macrophages were stained by immunohistochemistry (IHC) using a Vector kit (Vector Labs), following the manufacturer’s instructions. H & E slides were stained with hematoxylin (Harleco) for 8 min. Slides were rinsed in tap water, dipped in acid alcohol, and rinsed again. Slides were then dipped in ammonia water and rinsed in tap water for 4 min. Following multiple dips in 95% alcohol, slides were counterstained in eosin Y (1% alcoholic, Harleco). For IHC staining, antigen retrieval was achieved using trypsin digestion
Endogenous peroxidase activity was quenched by addition of 3% hydrogen peroxide for 10 min and washed in 1× PBS. Nonspecific binding was blocked using normal rabbit serum (Vector) for 30 min. Macrophages were then stained using F4/80 rat anti-mouse primary antibody (eBioscience) for 30 min and washed in 1× PBS. An isotype control slide was also stained with mouse IgG2a (DakoCyto- mation). A secondary rabbit anti-rat biotinylated antibody was diluted in PBS, incubated with diaminobenzidine (DAB) Pierce solution diluted 1:10, and washed in tap water. Slides were counterstained in hematoxylin (Harleco) for 10 min and rinsed in running tap water for 5 min. All slides were then dehydrated in a graded alcohol series, washed in xylene, and mounted with coverslips.

Statistics. All statistics were performed using GraphPad InStat 3 software. Survival data were analyzed by Kaplan-Meier test, whereas comparisons between and within groups were analyzed using 1-way ANOVA with Tukey-Kramer multiple comparisons. Mann-Whitney U-tests were used when data were not normally distributed. Statistical significance was accepted at \( P < 0.05 \).

Results

**AHCC increased survival and maintained weight of young mice after primary influenza infection.** Mice supplemented with AHCC exhibited 95% survival compared with 75% survival in control mice through d 10 postinfection (Fig. 1).

Importantly, there were no differences in the body weights of control (25.6 g) or AHCC-supplemented (25.0 g) mice at d −7 or at d 0 (26.0 g each). However, AHCC-supplemented mice lost less weight (\( P < 0.001 \)) and also recovered weight more quickly than control mice following infection (Fig. 2). AHCC-supplemented mice exhibited a maximal weight loss of 1.8 g (7% of initial body weight) at d 4, whereas control mice exhibited a maximal loss of 5.9 g (23%) at d 6–7 postinfection.

**Lung virus titers were reduced in mice supplemented with AHCC.** Virus was undetectable at baseline, and values postinfection were adjusted for background. Viral load in the lungs of control mice was higher than in AHCC-supplemented mice at d 5, 7, and 10 postinfection (Table 1, \( P < 0.05 \)).

**Supplementation with AHCC improved lung epithelial integrity following influenza infection.** At d 10, lungs from control mice exhibited erosion of the epithelium, whereas recovery was apparent in lungs from AHCC-supplemented mice (Fig. 3). Lung tissue from both control and supplemented mice showed cellular infiltration at d 10.

**Mice supplemented with AHCC demonstrated enhanced NK cell activity in the lung and spleen and increased NK cell percentage and number in lungs during influenza infection.** The kinetics of NK activity in response to influenza infection was altered in the lungs of AHCC-supplemented mice compared with those of control mice (Table 2). Whereas both groups demonstrated a peak in NK activity at d 3 postinfection (\( P < 0.001 \)), supplemented mice exhibited an increase in NK cytotoxicity at d 1 (\( P < 0.01 \)) and maintained a higher activity at d 4 than the control mice (\( P < 0.01 \)). Similarly, NK activity was significantly elevated in the lungs of AHCC-supplemented mice at d 7 and 10 compared with control mice (\( P < 0.05 \)).

**Table 1** Lung virus titers in control and AHCC-treated mice following influenza infection.

<table>
<thead>
<tr>
<th>Time post-infection (d)</th>
<th>Control (TCID(<em>{50}) log(</em>{10}))</th>
<th>AHCC (TCID(<em>{50}) log(</em>{10}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 3</td>
<td>1.83 ± 0.3</td>
<td>2.50 ± 0.0</td>
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<tr>
<td>d 5</td>
<td>4.25 ± 0.5**</td>
<td>1.50 ± 0.6</td>
</tr>
<tr>
<td>d 7</td>
<td>4.50 ± 0.0***</td>
<td>0.17 ± 0.3</td>
</tr>
<tr>
<td>d 10</td>
<td>2.50 ± 0.6*</td>
<td>0.5 ± 0</td>
</tr>
</tbody>
</table>

\(^1\) Values are means ± SEM, \( n = 3 \). Asterisks indicate different from AHCC at that time: *\( P < 0.05 \), ***\( P < 0.001 \).
increased in the spleens of supplemented mice at d 2 (Table 3, \( P < 0.01 \)). Alterations in NK activity were associated with a greater percentage (\( P < 0.05 \)) and absolute number (\( P < 0.01 \)) of NK1.1+ lymphocytes at d 2 in the lungs of AHCC-supplemented mice compared with the control group (Table 4). The percent and number of NK cells did not change in the spleens of mice from either group throughout the course of infection (data not shown).

**Mice supplemented with AHCC exhibited reduced lymphocyte and macrophage infiltration in lung during influenza infection.** The number of total lymphocytes (\( P < 0.01 \)), as well as the number of CD4+ and CD8+ T cells (\( P < 0.05 \)), were elevated in the lungs of control mice at d 7 postinfection (Table 5). Control mice also demonstrated an increased number in the number of macrophages infiltrating the lung at d 7, determined as CD11b+ cells (Table 6, \( P < 0.05 \)). Macrophage infiltration in lung was confirmed by IHC staining against F4/80 primary antibody (Fig. 4). The peak in lymphocyte and macrophage infiltration at d 7 postinfection corresponded with the height in the severity of infection in control mice, as indicated by maximal weight loss.

### Discussion

Young mice supplemented with AHCC daily before and during the course of influenza infection exhibited increased survival, enhanced NK activity in the lung and spleen, and rapid virus clearance from the lung, relative to young mice given a vehicle control. The importance of NK cells in controlling primary influenza infection in the lung prior to the initiation of a virus-specific immune response has been suggested (3–5,20). Upon activation, NK cells respond rapidly, peaking within the first few days of infection. By targeting and eliminating virus-infected cells, NK cell-mediated cytosis acts to eliminate the source of the replicating virus (2). Activated NK cells also produce IFN-γ, which further increases the cytotoxicity of NK cells and activates antigen-specific CD8+ cytotoxic T lymphocytes (CTLs) (21–23). Our data indicated that influenza-induced NK activity in lungs was enhanced by supplementation with AHCC, which was associated with the initiation of viral clearance and a significant decrease in lung virus titers, compared with control mice as early as d 5 postinfection. Although we did not evaluate the production of antigen-specific CTLs, our previous kinetic studies found a peak in CTL activity and viral load at d 7 postinfection that was not followed by viral clearance until d 10 (19). The adaptive immune response requires time to evoke antigen presentation, such as by macrophages and dendritic cells, and T cell proliferation, including an increase in both CD4+ and CD8+ T lymphocytes. In this study, lymphocyte and macrophage infiltration in lungs following infection peaked at d 7 in control mice, as expected, but was less pronounced in AHCC-supplemented mice. These observations suggest the possibility that the more robust NK response was associated with a decreased reliance on the adaptive immune response for viral clearance. Future studies will attempt to evaluate the effect of AHCC supplementation on adaptive immunity. Taken together, however, the current data suggest that an enhanced NK cell response in AHCC-supplemented mice may have contributed to a decreased susceptibility to influenza infection.

Although the exact mechanism by which AHCC boosts NK activity remains under investigation, we speculate that α-1,4-glucans are recognized by C-type lectins, such as Dectin-1 on NK cells, thus initiating innate immunity. C-type lectins are also expressed on other cell types, including macrophages, dendritic cells, and γδ-T cells, that may further influence NK cells and the innate immune response through the production of cytokines. IFN-α/β, for example, is a cytokine produced during infection that induces an antiviral state in uninfected cells, thus limiting

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>d 0</th>
<th>d 1</th>
<th>d 2</th>
<th>d 3</th>
<th>d 4</th>
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<td>% cytotoxicity</td>
<td></td>
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<td>Control</td>
<td>3.1 ± 1.1ab</td>
<td>1.8 ± 0.3</td>
<td>10.1 ± 1.3b</td>
<td>20.2 ± 0.7</td>
<td>48 ± 0.4ab</td>
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<td>AHCC</td>
<td>2.4 ± 1.4a</td>
<td>6.9 ± 0.1a**</td>
<td>12.1 ± 0.9b</td>
<td>21.2 ± 1.0b</td>
<td>8.8 ± 1.0a***</td>
</tr>
</tbody>
</table>

1 Values are means ± SEM, \( n = 3 \). Means for a group with superscripts without a common letter differ, \( P < 0.05 \). Asterisks indicate different from control at that time: **\( P < 0.01 \).

### Table 3

<table>
<thead>
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<th>d 0</th>
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<tr>
<td>% cytotoxicity</td>
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<tr>
<td>Control</td>
<td>5.6 ± 1</td>
<td>14.2 ± 3.2</td>
<td>10.4 ± 1.6</td>
<td>63 ± 1.2</td>
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<tr>
<td>AHCC</td>
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<td>13.6 ± 2.5ab</td>
<td>20.4 ± 3.6**</td>
<td>6.7 ± 0.5a</td>
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1 Values are means ± SEM, \( n = 3 \). Means for a group with superscripts without a common letter differ, \( P < 0.05 \). Asterisks indicate different from control at that time: **\( P < 0.01 \).

### Table 4

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<th>d 0</th>
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<tr>
<td>%</td>
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<tr>
<td>Control</td>
<td>13.6 ± 0.9</td>
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<td>AHCC</td>
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<td>18.0 ± 2.6</td>
<td>29.2 ± 5.6*</td>
<td>18.9 ± 0.7</td>
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</tbody>
</table>

1 Values are means ± SEM, \( n = 3 \) mice per group per d. Asterisks indicate different from control at that time: *\( P < 0.05 \), **\( P < 0.01 \).

### Table 5

<table>
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<th>d 2</th>
<th>d 3</th>
<th>d 4</th>
<th>d 5</th>
<th>d 6</th>
<th>d 7</th>
<th>d 10</th>
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<td></td>
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<tr>
<td>Control</td>
<td>12.7 ± 1.0a</td>
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<td>137 ± 44a</td>
<td>274 ± 43b</td>
<td>100 ± 35a</td>
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<tr>
<td>AHCC</td>
<td>8.5 ± 0.8</td>
<td>8.3 ± 1.2</td>
<td>123 ± 30</td>
<td>123 ± 67</td>
<td>62 ± 11</td>
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<tr>
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<td>49.2 ± 8.3a</td>
<td>21.2 ± 8.4a</td>
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<tr>
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<td>15.9 ± 4.1</td>
<td>27.1 ± 12</td>
<td>11.5 ± 3.3</td>
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<tr>
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<tr>
<td>AHCC</td>
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<td>13.7 ± 4</td>
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<td>7.3 ± 2.8</td>
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</table>

1 Values are means ± SEM, \( n = 3 \). Means for a group with superscripts without a common letter differ, \( P < 0.05 \).
Previous kinetic studies of cytokine production in bronchoalveolar fluid and lung homogenates after influenza infection in mice have shown early production of IFN-α/β before the initiation of an influenza-specific adaptive immune response in the lung (24). Along with Type I IFN, IL-12 and IL-18 are also produced early in the innate immune response and act synergistically to activate NK cells to produce IFN-γ (21,25–27). Previous reports suggest that AHCC influences the production of a variety of cytokines (10), including enhanced IL-12 (NK stimulatory factor) production by macrophages (28) and IFN-γ production by antigen-specific CD8+ T cells (14). Therefore, it is possible that the enhanced NK cytotoxicity in AHCC-supplemented mice was due to alterations in endogenous cytokine production, improving the ability of NK cells to become activated during primary infection. In addition to producing IL-12, macrophages are also potent producers of inflammatory cytokines in response to influenza infection, including TNF-α, IL-1β, and IL-6. While these cytokines play an essential role in viral clearance, they are also associated with inflammation, tissue damage, and symptoms of disease (9,24–26). In this study, AHCC-supplemented mice had less macrophage infiltration and better epithelial integrity in their lungs following infection than control mice. As such, further studies are required to address the potential influence of AHCC on cytokines involved in both NK activation and the inflammatory response to influenza infection.

Finally, both the young and the elderly are at an increased risk for morbidity and mortality associated with influenza infection. AHCC has previously been reported to increase the number of NK cells in aged mice (29), and future studies should determine whether AHCC supplementation may abrogate the age-associated decline in inducible NK activity. Additionally, mice and humans demonstrate multiple age-associated impairments in immunity (30,31), including a reduced CTL response to influenza infection (19,22) and a loss of antibody production in response to influenza vaccination (32). Given the ability of AHCC supplementation to enhance influenza-induced NK activity in young mice and the clear connection between the NK cell-mediated innate immune response to influenza infection and the activation of adaptive immunity, future investigations should consider the possibility that AHCC may mitigate certain aspects of immunosenescence in response to influenza. In summary, if our data can be extended to the human circumstance, we suggest that supplementation with AHCC, a natural bioactive dietary supplement, may provide a feasible approach to improving the immune response to viral infections, such as influenza.

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