

Natural Killer Cell Activation and Modulation of Chemokine Receptor Profile In Vitro by an Extract from the Cyanophyta *Aphanizomenon flos-aquae*

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ABSTRACT The present research was designed to study the effects of an extract from the edible cyanophyta *Aphanizomenon flos-aquae* on human natural killer (NK) cells. We have previously shown, using a double-blind randomized placebo-controlled crossover design, that ingestion of 1.5 g of dried whole *A. flos-aquae* resulted in a transient reduction in peripheral blood NK cells in 21 healthy human volunteers, suggesting increased NK cell homing into tissue. We have now identified an extract from *A. flos-aquae* (AFAe) that directly activates NK cells *in vitro* and modulates the chemokine receptor profile. NK cell activation was evaluated by expression of CD25 and CD69 on CD3⁺CD56⁺ cells after 18 hours. Changes in CXCR3 and CXCR4 chemokine receptor expression after 5–60 minutes were evaluated by immunostaining and flow cytometry. AFAe induced the expression of CD69 on CD3⁺CD56⁺ NK cells, induced CD25 expression on 25% of these cells, and acted in synergy with interleukin 2. NK cells enriched by RosetteSep[®] (StemCell Technologies Inc., Vancouver, BC, Canada) were not activated by AFAe, indicating that the NK activation was dependent on other cells such as monocytes. The low-molecular-weight fraction <5,000 of AFAe was responsible for the most robust NK cell activation, suggesting novel compounds different from previously reported macrophage-activating large polysaccharides.

KEY WORDS: • *Aphanizomenon flos-aquae* • blue-green algae • human • immunomodulation • low-molecular-weight peptides • trafficking

INTRODUCTION

THE EDIBLE CYANOPHYTA *Aphanizomenon flos-aquae* is known for its bioavailable antioxidants, including phycocyanin,¹ immunomodulatory polysaccharides,^{2,3} and a beneficial lipid profile.⁴ We have previously reported that ingestion of *A. flos-aquae* results in rapid and transient homing of natural killer (NK) cells *in vivo*.⁵ The current study was undertaken to explore several mechanisms that may contribute to an induction of NK cell trafficking and activation.

NK cells play a key role in immune surveillance and the primary defense against viral infections and cancer, as they are capable of killing virally infected cells and some tumor cells without prior sensitization.⁶ NK cells also contribute to the defense against some bacteria.⁷ This involves prior phagocytosis of bacteria by macrophages followed by contact between macrophages and NK cells.⁸ RNAs for Toll-like receptor (TLR) 1–6 and TLR9 are expressed in circulating NK cells,⁹ allowing NK cells to respond to various bacterial cell wall components, including peptidoglycans and lipopolysaccharides, as well as bacterial DNA.

Dietary intervention for enhancement of immune surveillance and primary defense includes nutritional support of a healthy composition of the gut flora. Commensal organisms in the gut, including lactobacilli, have been found to possess potent NK-activating properties.^{10–12} This property extends to fixed cells and isolated cell wall fractions of the commensal organisms.^{13–16}

Consumption of cyanobacteria as dietary supplements presents interesting possibilities in terms of NK cell activation. Cyanobacteria are primitive organisms resembling bacteria as well as chloroplasts and are thought to represent organisms that gave rise to chloroplasts.¹⁷ They share molecular similarities with bacteria, including bacterial DNA and cell wall components such as peptidoglycans, which are known to induce NK cell activation.¹³ In addition, the outer cell wall represents carbon storage and consists of large complex polysaccharides with immunomodulatory activity^{2,18} and may contribute to the documented antiviral¹⁹ and anticancer²⁰ effects of some blue-green algae. Consumption of edible cyanobacteria may thus present similar compounds to the immune system as some commensal gut bacteria.

The two most common edible cyanobacteria include *Spirulina* and *A. flos-aquae*. Within the class of Cyanophyta, these two genera belong to separate orders: *Spirulina* to the Oscillatoriales and *Aphanizomenon* to the Nostocales. Tax-

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onomic re-evaluation of *Aphanizomenon* strains concluded that previous reports on toxin-producing *Aphanizomenon* were based on misclassification of *Anabaena*, which is toxic.²¹ During certain seasons, including the late fall, the cyanophyta *A. flos-aquae* grows in almost complete monoculture in the hypereutrophic Upper Klamath Lake, Oregon, during which times the biomass can be harvested for human and animal consumption.²²

The data presented here show that the extract of *A. flos-aquae* (AFAe) is a potent activator of NK cells *in vitro*, with the highest activity found in the low-molecular-weight fraction.

MATERIALS AND METHODS

Algal extract

Harvested, wild-grown *A. flos-aquae* biomass was filtered, cooled, and dried using Refractance Window™ technology (Desert Lake Technology LLC, Klamath Falls, OR). Dried algal material was heated to 85°C in 10% ethanol for 3 hours. The supernatant was decanted and then precipitated by adding 50% ethanol. The precipitate was dried, giving a pale yellow powder, Migratose™ (Desert Lake Technology). An aqueous extract was prepared fresh prior to each *in vitro* experiment by weighing 0.2 g of powder into 2 mL of phosphate-buffered saline. This resulted in a deep orange liquid extract. Solids were removed by centrifugation, and the supernatant was sterile-filtered using a 0.22- μ m syringe filter. This extract was the stock solution for all *in vitro* experiments and is referred to as AFAe throughout this paper. Serial dilutions were prepared in cell culture medium. In some experiments, AFAe was further separated into high- and low-molecular weight compounds by applying the crude extract to Amicon Ultra centrifugal filter devices (Millipore, Billerica, MA) with a 5-kDa cutoff. Both the low-molecular-weight and the high-molecular-weight fractions were re-suspended in phosphate-buffered saline to the same volume as originally applied to the filter device, and serial dilutions were prepared.

Gel electrophoresis

The electrophoretic profile of AFAe was evaluated by gel electrophoresis by mixing AFAe 1:1 (vol/vol) in Laemmli sample buffer (Bio-Rad, Hercules, CA) without mercaptoethanol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 4–15% precast ReadyGels (Bio-Rad) in Tris/glycine/sodium dodecyl sulfate buffer (Bio-Rad) for 1 hour at 120 V. Staining of the gels for protein was performed using Coomassie Brilliant Blue G (Sigma-Aldrich, St. Louis, MO). Staining for both protein and carbohydrates was performed using a silver staining kit (Bio-Rad). Densitograms were prepared using ImageJ version 1.37 software (National Institutes of Health, Bethesda, MD).

Reagents and monoclonal antibodies

The following monoclonal antibodies directly conjugated with fluorochromes were purchased from Becton-Dickinson (San Jose, CA): CD3-peridinin-chlorophyll-protein (PerCP), CD14-phycoerythrin (PE), CD25-fluorescein isothiocyanate (FITC), CD45-FITC, CD56-FITC, CD56-PE, and CD69-FITC. Buffers including RPMI 1640 medium, Histopaque 1077, and phosphate-buffered saline were purchased from Sigma-Aldrich.

Purification of peripheral blood mononuclear cells (PBMCs)

Peripheral venous blood samples were obtained after informed consent from healthy human volunteers between the

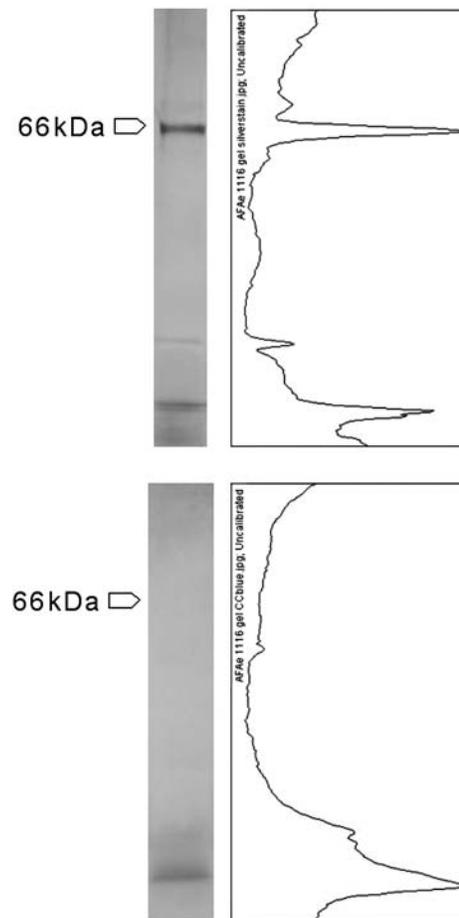


FIG. 1. Comparison of the electrophoretic band pattern of AFAe stained with either silver stain (**top**) or Coomassie Brilliant Blue (**bottom**). Densitograms are shown to the right of each lane. The silver stain revealed a band of approximately 60 kDa, which did not stain with Coomassie Brilliant Blue (CCblue), indicating a large polysaccharide. Both stains revealed a series of smaller bands below 8 kDa, indicating small peptides in AFAe. The data are representative of three separate experiments.

ages of 20 and 60 years. Heparinized whole blood was layered onto Histopaque 1077 and centrifuged for 25 minutes at 400 g. The PBMC-rich interface was harvested and washed twice in phosphate-buffered saline without calcium or magnesium.

Enrichment of NK cells

NK cells were enriched by removal of other cell types using the negative depletion kit RosetteSep® (StemCell Technologies Inc., Vancouver, BC, Canada), which contains an antibody cocktail towards CD3, CD4, CD19, CD36, CD66, and glycoprotein A. The RosetteSep cocktail was added directly to whole blood, allowing the antibodies to bind unwanted cells to erythrocytes. The blood was then applied to Histopaque 1077 and centrifuged for 25 minutes at 400 g. Only cells that were not recognized by the antibodies in the cocktail remained in the interface above the Histopaque. This allowed the harvest of highly enriched NK cells (90% pure).

Induction of cell surface markers

For the testing of activation markers CD69 and CD25, freshly purified PBMCs were resuspended in culture

medium and exposed to serial dilutions of AFAe for 18 hours. For the testing of chemokine receptor CXCR3 and CXCR4 expression, similar cultures were established and incubated for 5, 15, and 30 minutes. Cells were washed in phosphate-buffered saline containing 1% bovine serum albumin and 0.02% sodium azide. Cells were resuspended in 50 μ L of buffer. Monoclonal antibodies were added and incubated in the dark at room temperature for 10 minutes. An additional 110 μ L of buffer was added to each well, and the plates were washed. Supernatant was discarded, and the cells were resuspended in 50 μ L of buffer and transferred to 0.4 mL of 1% formalin. Samples were stored dark and acquired by flow cytometry within 4 hours. Acquisition was performed using a FACScalibur™ flow cytometer and CellQuest™ software (both from Becton-Dickinson). Analysis of fluorescence intensity of each marker was performed by electronic gating on CD3⁻CD56⁺ NK cells as well as on CD3⁺CD56⁺ NKT cells, using the FlowJo software (Tree Star Inc., Ashland, OR).

Interferon- γ (IFN- γ) enzyme-linked immunosorbent assay (ELISA)

The production of IFN- γ in culture supernatants was evaluated using a commercial ELISA kit (R & D Systems

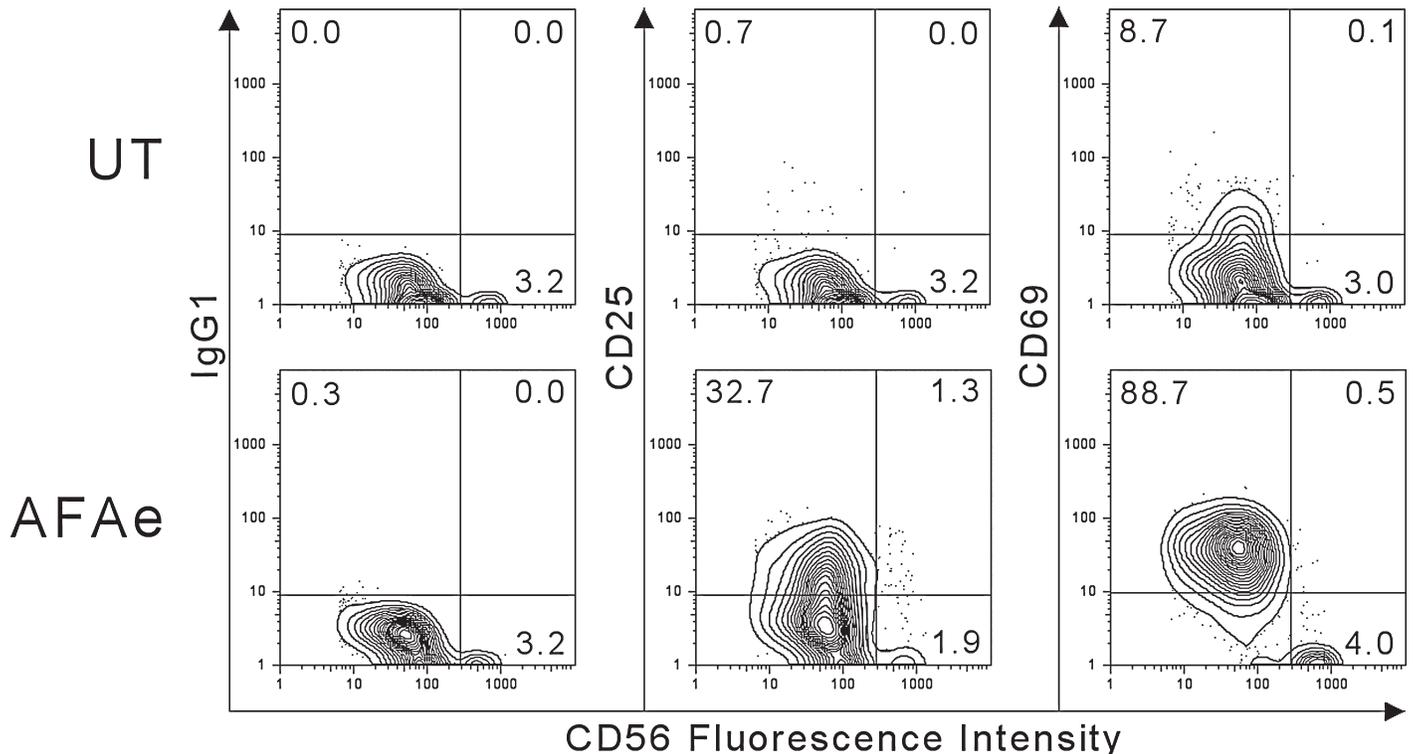


FIG. 2. AFAe induced the expression of the IL-2 receptor CD25 (center panels) and the CD69 activation marker (right panels) on almost all CD3⁻CD56^{dim} NK cells but not on the CD3⁻CD56^{bright} NK cells. (Left panels) Isotype control plots. Contour plots show data from (top row) untreated (UT) cells and (bottom row) AFAe-treated samples. The contour plots display data on CD3⁻CD56⁺ lymphocytes. The two-dimensional plot allows evaluation of CD56 intensity versus expression of the activation marker CD69. The data shown are representative of three separate experiments using cells from three different donors.

Inc., Minneapolis, MN). Supernatants were tested in triplicate from 5-day cultures where PBMCs had been exposed to AFAe and compared to untreated samples (negative controls) and phytohemagglutinin-treated samples (positive controls). Microplates were read on a BioTek (Winooski, VT) Powerwave microplate reader. Data were exported into Microsoft Excel (Microsoft Corp., Redmond, WA), where averages and standard deviations for each set of triplicate sample were calculated.

Statistical analysis

Statistical analysis was performed using Microsoft Excel. Statistical significance was tested using Student's *t* test, with a value of $P < .05$ indicating a significant difference between treatments.

RESULTS

Electrophoretic pattern for protein and carbohydrate content in AFAe

The extract AFAe was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and parallel gels were developed with either the protein stain Coomassie Brilliant Blue or silver stain, which also stains carbohydrates. The band patterns and densitometry are shown in Figure 1. A distinct pattern was seen, revealing the presence of two different groups of compounds in the extract. A strong band around 60 kDa was visible after silver staining but was completely absent after colloidal Coomassie Brilliant Blue staining, indicating the presence of larger polysaccharides in the extract. Several small compounds stained with both stains in a similar pattern, indicating the presence of several smaller peptides in the extract. The crude extract was further separated by centrifugation over a centrifugal filter device with a cutoff at 5 kDa, and the two fractions were tested in parallel to crude AFAe on NK cell activation *in vitro* (see Fig. 3).

CD69 induction: enhancement of interleukin (IL-2) but partial inhibition of phytohemagglutinin on both NK and NKT cells

The incubation of PBMCs overnight with AFAe resulted in a strong induction of CD69 and moderate induction of CD25 expression on CD3⁻CD56^{dim} NK cells, whereas no induction of CD69 or CD25 was seen on CD3⁻CD56^{bright} NK cells (Fig. 2).

TABLE 1. AFAE INDUCTION OF THE CD69 ACTIVATION MARKER ON NK CELLS

Treatment	CD69 mean fluorescence intensity	
	NK cells from PBMCs	Enriched NK cells
Negative control	5.18 ± 1.13	2.14 ± 0.07
AFAe	8.37 ± 0.52	2.70 ± 0.08

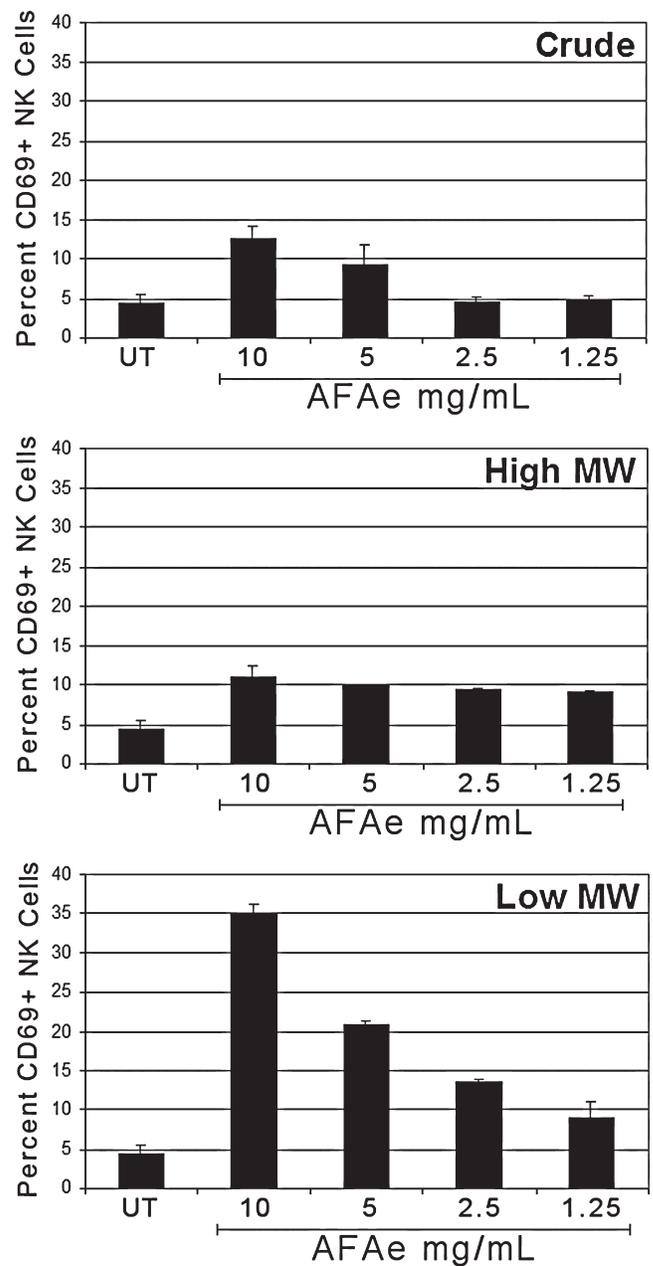


FIG. 3. Induction of the activation marker CD69 on CD3⁻CD56⁺ cells by either (top panel) crude AFAe or the high- (middle panel) and low- (bottom panel) molecular weight (MW) fractions after separation over a centrifugation filtration device with a 5,000 cutoff. The high MW fraction contained compounds larger than 5,000, which included the larger polysaccharide seen in Figure 1. The low MW fraction contained the smaller peptides. The low MW fraction had the highest NK-activating properties. The data are representative of three separate experiments. UT, untreated.

NK activation by AFAe required the presence of other cells

In order to examine whether the NK activation by AFAe was a direct effect on NK cells or was dependent on monocytes or T cells in the PBMC cultures, we compared the in-

TABLE 2. SYNERGISTIC EFFECTS BY AFAE AND IL-2^a

Treatment	CD69 expression (mean fluorescence intensity)	IFN- γ production (pg/mL)
Negative control	2.91 \pm 0.11	120 \pm 60
AFAe	10.16 \pm 0.39	422 \pm 118
AFAe + IL-2	27.63 \pm 1.76*	4,090 \pm 170*
IL-2	17.18 \pm 0.17	2,690 \pm 1,927

^aThe dose of IL-2 used was 50 International Units/mL.

The increase in response between the treatments with IL-2 alone compared to AFAe + IL-2 was statistically significant with * $P < .05$.

duction of CD69 expression in parallel cultures of PBMCs versus enriched NK cells from the same sample of PBMCs. A positive control was used to verify that the functionality of NK cells was not compromised by the enrichment protocol. AFAe induced CD69 expression on NK cells from PBMC cultures but not on enriched NK cells (Table 1).

The low-molecular-weight fraction induced strong NK activation as compared to the high-molecular-weight fraction of AFAe

The electrophoretic properties of AFAe showed two major groups of compounds: larger polysaccharides and lower-molecular-weight peptides. These were separated using a centrifugation filter device. PBMC cultures were treated with crude AFAe, AFAe high molecular weight, or AFAe low molecular weight and incubated for 18 hours to allow for CD69 induction. The percentage of NK cells expressing CD69 after each treatment was evaluated. Treatment with crude AFAe at 10 mg/mL resulted in activation of 12–14% NK cells, with a dose–response that rapidly decreased to baseline levels (Fig. 3, top panel). The high-molecular-weight fraction of AFAe resulted in a similar level of activation of NK cells (Fig. 3, middle panel). In contrast, the low-molecular-weight fraction of AFAe resulted in a substantially higher activation of NK cells than either crude AFAe or AFAe high molecular weight (Fig. 3, bottom panel). This indicated that the small peptides possess potent NK-activating properties different from the larger polysaccharides with macrophage-activating properties as reported by Pasco and co-workers.^{2,3}

Induction of IFN- γ production and synergy with IL-2

The induction of the activation marker CD69 on CD3⁻CD56⁺ NK cells by AFAe was assayed in the context of IL-2 (50 international units/mL). The intensity of CD69 expression after AFAe treatment alone was significantly above the baseline expression seen on untreated cells. IL-2 triggered higher expression of CD69 than AFAe alone and acted in synergy with AFAe such that when AFAe was added immediately prior to IL-2, a higher CD69 expression level was seen than with IL-2 alone (Table 2).

Treatment of PBMCs with AFAe for 5 days *in vitro* resulted in an increase in IFN- γ in the culture supernatants (Table 2). When the cultures were treated with both AFAe

and IL-2, the level of IFN- γ production was higher than with either AFAe or IL-2 alone, indicating that the synergy seen between AFAe and IL-2 for CD69 induction extends to IFN- γ production *in vitro*.

Down-regulation of the chemokine receptors CXCR3 and CXCR4

The chemokine receptors CXCR4 and CXCR3 are expressed on a proportion of NK cells. The exposure of cultures to AFAe for 30 minutes resulted in down-regulation of the expression of these two chemokine receptors on both NK cells and NKT cells (Fig. 4).

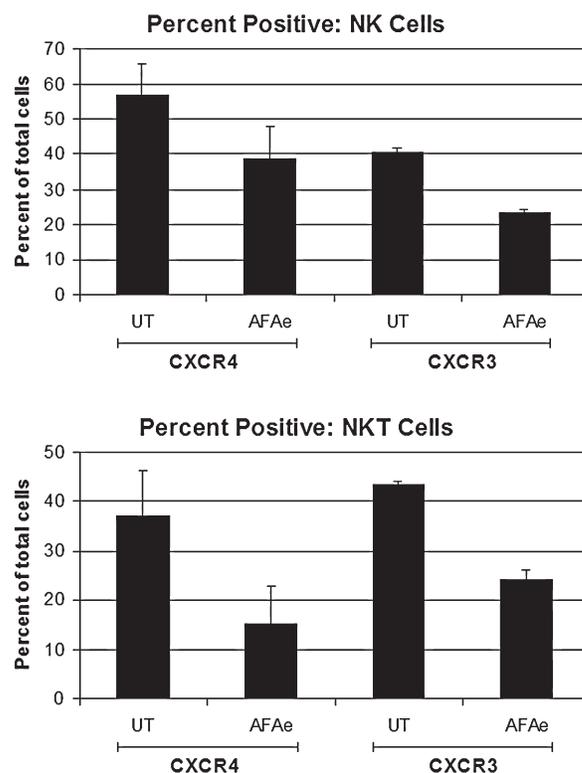


FIG. 4. Expression of the chemokine receptors CXCR3 and CXCR4 for NK (top histogram) and NKT (bottom histogram) cells. PBMCs were incubated for 30 minutes in the presence of AFAe. Untreated (UT) cultures served as a control. Flow cytometric analysis included electronic gating on CD3⁻CD56⁺ NK cells and CD3⁻CD56⁺ NKT cells.

DISCUSSION

In this study, we have shown that AFAe, an extract from whole dried *A. flos-aquae* biomass, rapidly changes the chemokine receptor profile of NK cells *in vitro*. We have previously shown that consumption of 1.5 g of whole *A. flos-aquae* results in a transient decrease of circulating NK cells, using a randomized double-blinded placebo-controlled design involving 21 healthy subjects.⁵ The present study shows that a specific extract from *A. flos-aquae* possesses the ability to activate NK cells *in vitro*, as reflected by the induction of CD69 expression. The NK-activating effect appears to be dependent on other cell types, since the effect was not seen on RosetteSep-enriched NK cells. AFAe also produced a significant reduction in the expression of both CXCR3 and CXCR4 on peripheral blood NK cells. The chemokine receptor CXCR4 is specific for stromal cell-derived factor-1 and plays a role in recruiting NK cells into the bone marrow environment. Other chemokines, including IL-8 and fractalkine, are involved in recruiting CD16⁺ NK cells into nonlymphoid tissue,²³ and CCR5 is involved in homing to the liver.²⁴ The CD16⁺ cells correspond to the CD56^{dim} cells, which are the cells responding to AFAe in our study. We suggest that while the down-regulation of CXCR4 may reduce the NK cell homing to the marrow environment, the cells may become more sensitive to other chemotactic signals, thereby possibly increasing homing as part of immune surveillance of tissue other than marrow. Given that the effect *in vivo* is seen after oral consumption, it is tempting to suggest that the gut mucosal-associated lymphoid tissue would be affected first, possibly initiating a cascade of events such as macrophage activation and induction of chemotactic signals.

Further chemical characterization of the components of AFAe is needed; however, the large polysaccharide may likely be of cell wall origin. The large polysaccharide has a different molecular weight than the complex polysaccharide isolated from *A. flos-aquae* by Pasco and co-workers.^{2,3} The small peptides with NK-activating properties are most likely to represent cell wall peptidoglycan breakdown products, capable of triggering signaling via TLRs.^{25,26} The observation that NK activation depended on other cells in the cultures could suggest that a cross-talk between monocytes and NK cells is involved. It has been shown that NK cells constitutively express α -defensin, which is rapidly released after TLR-mediated signaling.²⁷ Interestingly, mammalian defensins have the ability to act as chemokines and participate in lymphocyte recruitment.^{28,29} The *in vivo* effect of AFAe consumption on NK cells could involve a cascade of events in which TLR-mediated signaling, triggered by proteoglycans, and *A. flos-aquae*-derived defensin molecules trigger initial events, leading to rapid amplification locally in the gut. The overall *in vivo* effect of AFAe consumption must take into account not only the direct NK-activating properties, but also the effect of AFAe on other cell types, including macrophages. Further work is needed to evaluate to what extent consumption of AFAe may contribute to increased protection against viral disease.

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During the time this study was conducted, the co-author Christian Drapeau held the position of director for Research & Development at Desert Lake Technologies LLC, a harvester of *A. flos-aquae*. No other co-authors have any financial interest in the subject matter.

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