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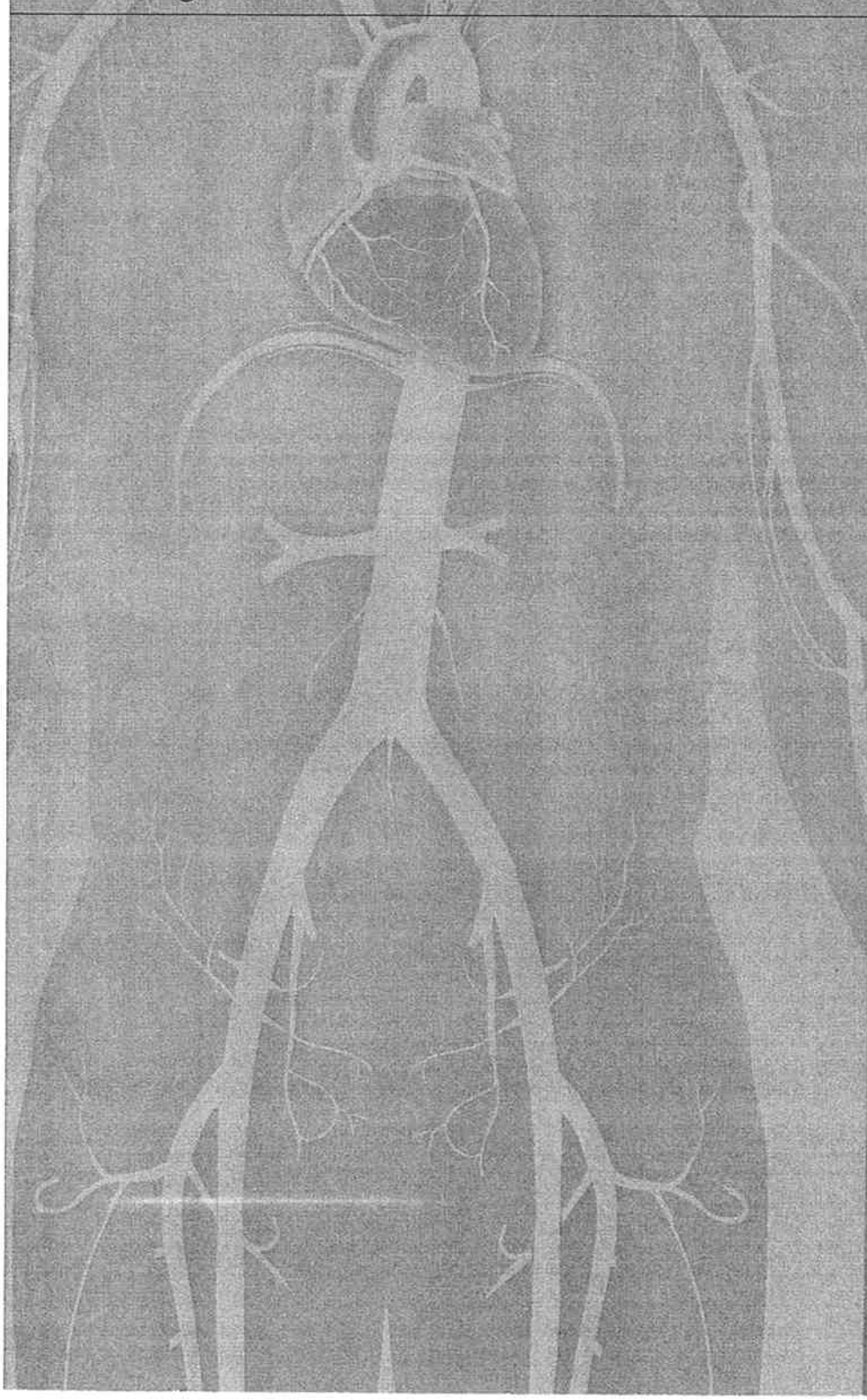

Mobilization of Human $CD34^+CD133^+$ and $CD34^+CD133^-$ Stem cells in Vivo by Consumption of an Extract from *Aphanizomenon flos-aquae*—Related to Modulation of CXCR4 Expression by an L-Selectin Ligand?

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Mobilization of human $CD34^+CD133^+$ and $CD34^+CD133^-$ stem cells in vivo by consumption of an extract from *Aphanizomenon flos-aquae*—related to modulation of CXCR4 expression by an L-selectin ligand?☆

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Abstract

Objective: The goal of this study was to evaluate effects on human stem cells in vitro and in vivo of an extract from the edible cyanobacterium *Aphanizomenon flos-aquae* (AFA) enriched for a novel ligand for human CD62L (L-selectin).

Experimental approach: Ligands for CD62L provide a mechanism for stem cell mobilization in conjunction with down-regulation of the CXCR4 chemokine receptor for stromal derived factor 1. Affinity immunoprecipitation was used to identify a novel ligand for CD62L from a water extract from AFA. The effects of AFA water extract on CD62L binding and CXCR4 expression was tested in vitro using human bone marrow $CD34^+$ cells and the two progenitor cell lines, KG1a and K562. A double-blind randomized crossover study involving 12 healthy subjects evaluated the effects of consumption on stem cell mobilization in vivo.

Results: An AFA extract rich in the CD62L ligand reduced the fucoidan-mediated externalization of the CXCR4 chemokine receptor on bone marrow $CD34^+$ cells by 30% and the $CD62L^+$ $CD34^+$ cell line KG1A by 50% but did not alter the CXCR4 expression levels on the $CD34^-$ cell line K562. A transient, 18% increase in numbers of circulating $CD34^+$ stem cells maximized 1 hour after consumption ($P<.0003$). When 3 noncompliant volunteers were removed from analysis, the increase in $CD34^+$ cells was 25% ($P<.0001$).

Conclusion: AFA water extract contains a novel ligand for CD62L. It modulates CXCR4 expression on $CD34^+$ bone marrow cells in vitro and triggers the mobilization of $CD34^+$ $CD133^+$ and $CD34^+$ $CD133^-$ cells in vivo.

Keywords: L-selectin; Ligand; Human; Adult stem cell; CD34; CD133; KG1a; K562; Bone marrow; Mobilization; Blue-green algae; Cyanobacteria; *Aphanizomenon*; In vivo; In vitro

Abbreviations: AFA, *Aphanizomenon flos-aquae*; PBMC, Peripheral blood mononuclear cells; PMN, Polymorph-nucleated cells.

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1. Introduction

Much recent research has focused on the role of selectins and their ligands in mobilization of bone marrow stem cells. L-selectin belongs to the selectin family of cell adhesion molecules involved in cellular migration during normal immunosurveillance and inflammatory conditions. L-selectin is best known as a homing molecule for recirculating lymphocytes to recognize high endothelial venules during the process of extravasation [1–3] and for leukocytes to recognize and home to inflamed tissues [4–8]. However, L-selectin plays significant roles in other physiological cell adhesion processes as well, including the retention vs. release of bone marrow stem cells into the blood circulation [9–11].

Of special importance are findings that engagement of L-selectin by some ligands will modulate the expression of the CXCR4 chemokine receptor [12]. The CXCR4 receptor specifically recognizes the chemokine stromal derived factor 1 (SDF-1), which acts as a potent chemoattractant for stem cells and assists in retaining stem cells within the bone marrow environment [13–16]. The chemoattractant properties of SDF-1 on stem cells were shown in vitro [17] as well as in vivo to be directly associated with recruitment of stem cells into kidney [18] and liver [19]. The mobilization of recruitment of stem cells is associated with repair of the central nervous system [20,21], heart [22,23], and other tissues [24]. Stem cell mobilization and homing involve a series of G-protein-coupled receptors that can interact with each other as well as with adhesion molecules [25,26]. It is proposed that loss of responsiveness towards CXCR4 may be one of several contributing mechanisms that allow some bone marrow stem cells to detach and leave the bone marrow as part of the mobilization process [27,28].

The use of selectin ligands has been proposed as a mechanism for stem cell mobilization [29]. Some L-selectin ligands (LSLs), including fucoidan and sulfatide, have a proven effect on stem cell mobilization [29,30]. The mobilization appears to happen in selectin-dependent and -independent mechanisms in tandem. As an example, the sulfated polysaccharide fucoidan can act as an LSL and up-regulate the chemokine receptor CXCR4, a receptor for SDF-1. However, fucoidan also assists stem cell detachment within the marrow by binding to another adhesion receptor, CD11b, during stem cell mobilization [30].

The objective of this study was to evaluate the effects on human stem cells in vitro and in vivo of an extract from *Aphanizomenon flos-aquae* (AFA), enriched for a novel ligand for human L-selectin. We report here that a novel compound from the blue-green algae AFA binds to the ligand-binding area of human L-selectin. The effect of this compound was tested in various in vitro assays as well as on stem cell mobilization in humans.

2. Materials and methods

2.1. Buffers and media

For cell cultures, freshly isolated human marrow cells, as well as the KG1a and K562 cell lines, were resuspended and cultured in RPMI-1640 with 10% fetal calf serum (Gibco, Grand Island, NY, USA), 1% penicillin and streptomycin, and L-glutamine. For immunostaining, cells were washed, resuspended, and stained in phosphate-buffered saline (PBS) containing 0.02% azide and 1% fetal calf serum or bovine serum albumin.

2.2. Cyanobacterial extracts

Dried powder of the freshwater blue-green algae AFA was obtained from Desert Lake Technologies, Keno, OR, USA. Dried powder of *Spirulina platensis* was obtained from Healthforce Nutritionals, Escondido, CA, USA. One gram of dried algal material was resuspended in 10 ml PBS and incubated for one hour at 4°C and protected from light. The resulting slush was mixed by repeated inversion of the vial and centrifuged at 400 g for 10 min. The bright blue supernatant was decanted and sterile-filtered using a 0.22-mm filter. This filtrate of AFA water extract, AFA-W, was stored cold and dark and used within the same day of preparation.

2.3. Monoclonal antibodies

The CD62L monoclonal antibody TQ1 (specific for the ligand-binding area of the L-selectin molecule) linked to phycoerythrin (PE) was purchased from Coulter (Hialeah, FL, USA). CD45-PerCP, CD11b-PE, CD14-PE, and isotype control antibodies were obtained from Becton–Dickinson (San Jose, CA, USA). Monoclonal antibodies for CXCR4 (clone 12G5) and CCR9 were obtained from R&D Systems (Minneapolis, MN, USA).

2.4. Capturing of ligand using Dynabeads and chimera proteins

In order to identify the molecular weight of the L-selectin binding compound, we used a cell-free method in which Dynabeads (Dynal Biotech, Lake Success, NY, USA) coated with protein G were incubated with an L-selectin chimera protein (R&D Systems). The chimera protein is a fusion of the extracellular domain of human L-selectin with the F_c portion of human immunoglobulin G (IgG), thereby facilitating binding to protein G. The chimera protein was captured and subsequently covalently linked to the protein G-coated Dynabeads using the protocol recommended by the manufacturer. Beads were incubated for 1 h in a freshly made 5.4-mg/ml solution of dimethyl pimelimidate × 2HCl (Sigma Aldrich, St Louis, MO, USA) in 0.2 M triethanolamine buffer (pH 8.0)

(Sigma Aldrich). The cross-linking was stopped by removing the beads from the cross-linking solution and resuspending them in 50 mM TRIS buffer (pH 7.5) (Sigma Aldrich) for 15 min. Unbound chimera was eluted off the beads by two washes in citrate/citric acid buffer (pH 2.8). The beads were then washed several times in PBS (pH 7.4), and added to a freshly made AFA water extract. Bound material from the AFA water extract was eluted in one of three ways: (1) boiling in Laemmli buffer containing beta-mercaptoethanol, (2) pH 12.5, or (3) competition for the LSL binding site using heparin. In parallel experiments, beads coated with recombinant human L-selectin/IgG1 fusion protein were used to see whether a similar water extract from another blue-green algae, *S. platensis*, contained a similar selectin-binding compound.

2.5. Electrophoresis

Samples of elutant from the Dynabead affinity method were prepared for gel electrophoresis by mixing 1:1 v/v in Laemmli sample buffer (BioRad cat# 161-0737) with mercaptoethanol. Sodium dodecyl sulfate (SDS) gel electrophoresis was performed on 4–15% gels (BioRad) in TRIS/glycine/SDS buffer (Biorad cat# 161-0732) for 1 h at 120 V. Electrophoresis for native protein was performed with SDS-free reagents, using native sample buffer (BioRad cat# 161-0738) for loading and TRIS/glycine buffer (BioRad cat# 161-0734) for electrophoresis.

2.6. Human subjects

Peripheral venous blood samples were obtained from healthy human volunteers between 20 and 45 years of age upon informed consent. Freshly drawn marrow was obtained upon informed consent, approved by the Merle West Medical Center Institutional Review Board (FWA 00002603). Blood and bone marrow samples were obtained under aseptic conditions and processed immediately.

2.7. Immunostaining for L-selectin

Polymorph-nucleated cells (PMN) cells were purified by gradient centrifugation, washed twice in PBS, and distributed into wells in a V-bottom 96-well microtiter plate at the concentration of 10^5 cells per well. Serial dilutions of freshly prepared AFA-W were added to the cells in the presence of sodium azide to inhibit cytoskeletal movement and block L-selectin shedding. Cells were incubated at room temperature and in the dark for 20 min. Cells were washed twice and resuspended in a volume of 50 μl PBS containing 1% fetal calf serum and 0.05% azide. Staining was performed with the TQ1-RD monoclonal antibody for 10 min; cells were washed, resuspended in 50 μl buffer, and fixed in 1% formalin. Samples were kept cold and dark until acquisition by flow cytometry. Acquisition was performed within 24 h of fixation.

2.8. Immunostaining for CXCR4 expression on different types of progenitor cells

The binding of fucoidan to L-selectin results in externalization of premade CXCR4 onto the cell surface. This is followed by internalization, creating a window of time for responsiveness to chemotactic factors. We used this system to examine whether AFA-W would compete with fucoidan for binding to L-selectin on the leukocyte cell surface and to assess whether it would block the externalization of CXCR4 triggered by fucoidan. To do so, freshly purified human bone marrow peripheral blood mononuclear cells (PBMC), as well as KG1a and K562 cells, were resuspended in RPMI at 10^6 cells per milliliter and distributed in a series of round-bottom microwells. Fucoidan was added to one series of wells, AFA-W to another series, and a mixture of fucoidan and AFA-W to the third series of wells. At different time points (1, 10, 20, 30, 40, 60 min), PBS containing sodium azide was added to wells in order to stop cytoskeletal movements and thereby stop the recycling of CXCR4. This allowed us to stain for CXCR4 expressed at the cell surface at each time point. Cells were washed in PBS containing sodium azide, stained with CXCR4-PE and CD34-fluorescein isothiocyanate (FITC) using the staining protocol described above, fixed in formalin, and acquired by flow cytometry. Analysis was performed by gating on the lymphocyte population using the forward and side scatter properties, then gating on the $CD34^+$ cells and analyzing the $CD34^+$ bone marrow-derived stem cells (BMSC) for their mean fluorescence intensity, which is proportional to their CXCR4 expression.

2.9. Induction of CXCR4 expression on various types of stem and progenitor-type cells

Using the same method as described above for BMSC, we evaluated the effects of fucoidan and AFA-W on the two cell lines KG1a and K562, both obtained from American Type Culture Collection, Manassas, VA, USA. The KG1a cell line is strongly positive for the stem cell marker CD34 and is phenotypically and functionally less mature than the parent cell line KG-1. KG1a is characterized as a promyeloblast cell line but does not spontaneously differentiate into more mature myeloid cells. The K562 cell line is also characterized as a highly undifferentiated, multipotential hematopoietic cell line but is negative for CD34 and does spontaneously differentiate into progenitors for erythrocytoid and myeloid cell types. Both cell lines were maintained in log phase, washed in PBS, resuspended in RPMI-1640, and used in the CXCR4 expression assay.

2.10. Study design for in vivo testing of consumption of an LSL-rich fraction of AFA

Two consumables were tested: StemEnhance (StemTech HealthSciences, San Clemente, CA, USA) and placebo. StemEnhance is a proprietary blend of the cytoplasmic and

cell wall-rich fractions of the whole plant biomass, enriched approximately fivefold in content of the LSL compared to the raw AFA biomass. One gram of StemEnhance or placebo was given to volunteers with 4–6 oz water. The appearance of the placebo was identical to that of the StemEnhance and consisted of green-dyed, finely ground potato flakes encapsulated in vegetable capsules. The following exclusion criteria were used: under 20 or over 65 years of age, pregnancy, severe asthma and allergies requiring daily medication, any known chronic illness or previous/current venereal disease, frequent recreational drug use, and impaired digestive function (including previous major gastrointestinal surgery). Twelve volunteers were scheduled on two study days one week apart. Testing was always performed at the same time of the day (8–11 a.m.) to minimize the effect of circadian fluctuations. Due to the interference from stress with the release vs. homing of other types of lymphocytes [31], effort was taken to minimize any physical and mental stress during testing. In addition, on each study day, volunteers were instructed to complete a questionnaire aimed at determining any exceptional stress-related circumstances that might affect the person on that particular study day. Predetermined criteria for exclusion from final analysis included significant lack of sleep and severe anxiety. After completing the questionnaire, volunteers were instructed to remain quiescent for 3 h, comfortably seated in a chair. After the first hour, the baseline blood sample was drawn. Immediately after drawing the baseline sample, a consumable was provided. Blood samples were later drawn 30, 60 and 120 min after ingestion of the consumable. At each time point, 5 ml of blood was drawn into heparin, and 2 ml blood was drawn into EDTA. The blood vials were placed on a rocking plate until use. The blood drawn into EDTA was used for obtaining a complete blood count (CBC) with differential, using a Coulter counter (Micro Diff II, Beckman Coulter). All CBCs were performed within an hour of drawing the sample. All CBCs were performed in triplicate. The heparinized blood was used for purification of the PBMC fraction by gradient centrifugation and processed for immunostaining and flow cytometry. The stem cell markers CD34-FITC (clone 8G12, BD BioSciences, San Jose, CA, USA) and CD133-PE (Miltenyi Biotech, Auburn, CA, USA) were used for two-color immunofluorescence. Staining of all samples with CD34-FITC/CD133-PE was performed in triplicate. IgG1-FITC and IgG1-PE isotype controls (BD BioSciences) were used in parallel samples. Separate, positive control samples for each donor included CD45-FITC and CD14-PE. Stained PBMC were fixed in 1% formalin and acquired by flow cytometry immediately. Files of 200,000 events were collected on each triplicate sample. The percent $CD34^+$ $CD133^-$, $CD34^+$ $CD133^+$, and the $CD34^-$ $CD133^+$ subsets were analyzed separately and were analyzed again after multiplying with the lymphocyte cell counts, as obtained from the average of the triplicate lymphocyte counts obtained by the CBC differential count.

2.11. Statistical analysis

Flow cytometry data from the in vitro analysis of phenotypical and functional changes were analyzed by CellQuest Pro (BD BioSciences) and FlowJo (Tree Star, Ashland, OR, USA). These data were exported to Microsoft Excel for further analysis, including Student's t test. Data were considered significant at $P<.05$.

In the human in vivo assay, for each volunteer, the time 0 (preingestion) level of $CD34^+$ cells for a given treatment (placebo or StemEnhance) was subtracted from the levels of $CD34^+$ cells in the samples collected at 30, 60 and 120 min post ingestion. Thus, the data used for analysis are normalized to each person's baseline. These data are repeated measures on a person, as well as repeated measures for treatment and time. The two trial factors were volunteer ID (number code) and analysis replicate. Normality of the dependent data was determined by the Shapiro–Wilk test. The data were analyzed using repeated measures analysis of variance, followed by contrast tests to compare placebo and StemEnhance at 30, 60 and 120 min. Significance was declared at $P<.05$. Analyses were carried out using Systat 11.01 (Systat, Richmond, CA, USA).

3. Results

3.1. AFA contains a ligand for human L-selectin (CD62L)

Using a cell-free affinity purification system in which paramagnetic Dynabeads were coated with a human L-

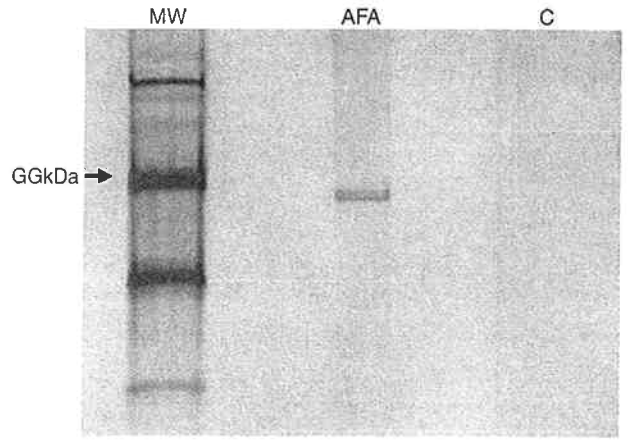

Fig. 1. SDS gel electrophoresis on eluted material after immunoprecipitation of an LSL from AFA is shown in the center lane labeled "AFA." This ligand was affinity-purified from AFA-W by paramagnetic Dynabeads covalently linked with the fusion protein rHuL-selectin/IgG F_c chimera and eluted from the beads by alkaline treatment at pH 12. The eluted material was subjected to SDS gel electrophoresis under reducing conditions. The negative control, as shown in the lane labeled "C," was prepared by incubating the Dynabeads coated with the fusion protein rHuL-selectin/IgG F_c chimera with PBS instead of AFA-extract. The molecular weight standards included bovine serum albumin with a molecular weight at 66 kDa, as indicated by the arrow in the lane labeled "MW." Two bands are seen with apparent molecular weight of 57 and 54 kDa, respectively. The data shown are representative of 12 independent experiments.

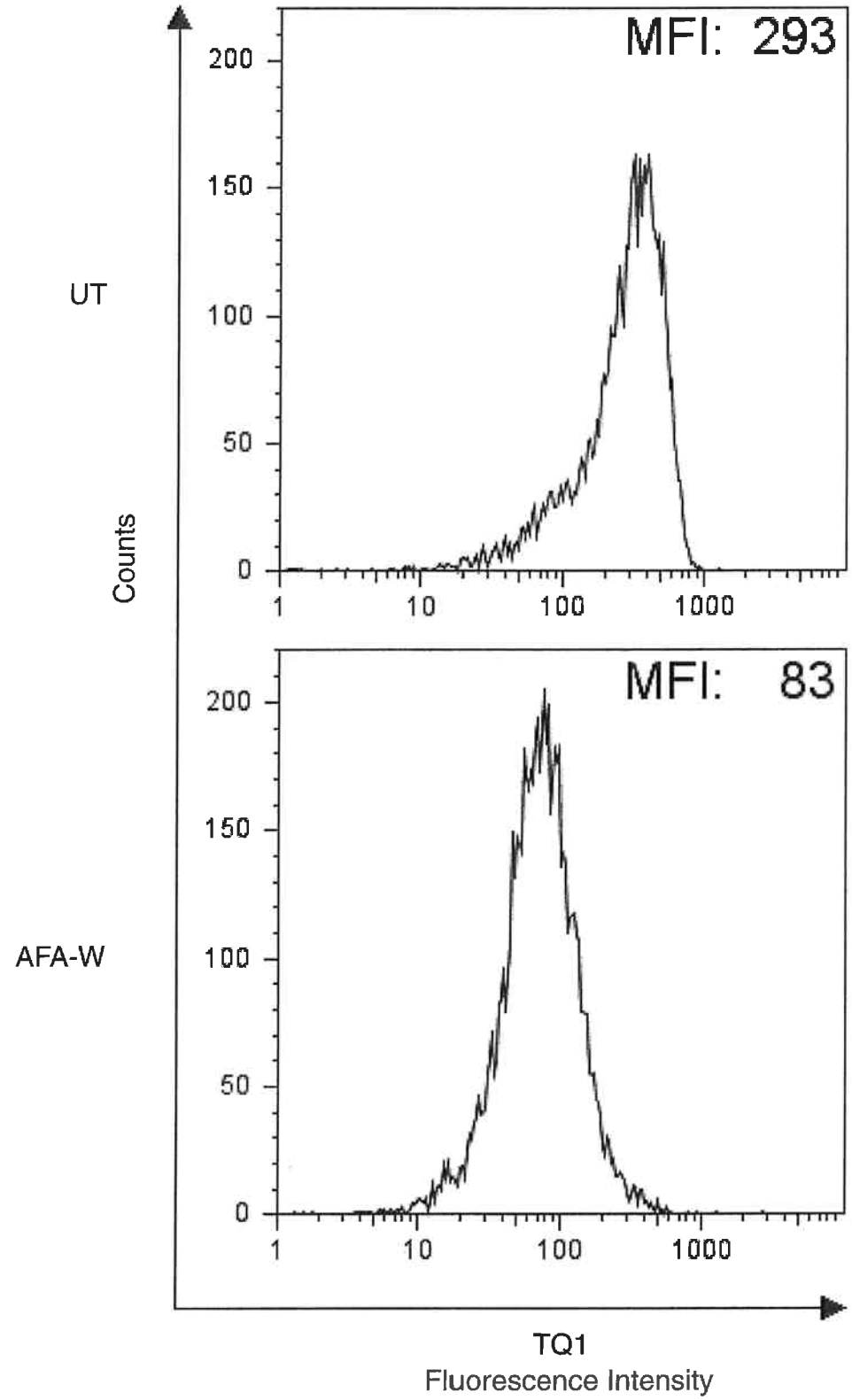

Fig. 2. Competition for the LSL binding site between the monoclonal antibody TQ1 against the compound from AFA. The top histogram shows the fluorescence intensity of TQ1 staining of PMN in the absence of competition for L-selectin binding (UT, untreated). The bottom histogram shows the reduction in TQ1 binding in the presence of AFA-W extract. The mean fluorescence intensity (MFI) of TQ1 staining on the PMN is shown for each histogram. The data are representative of five separate experiments.

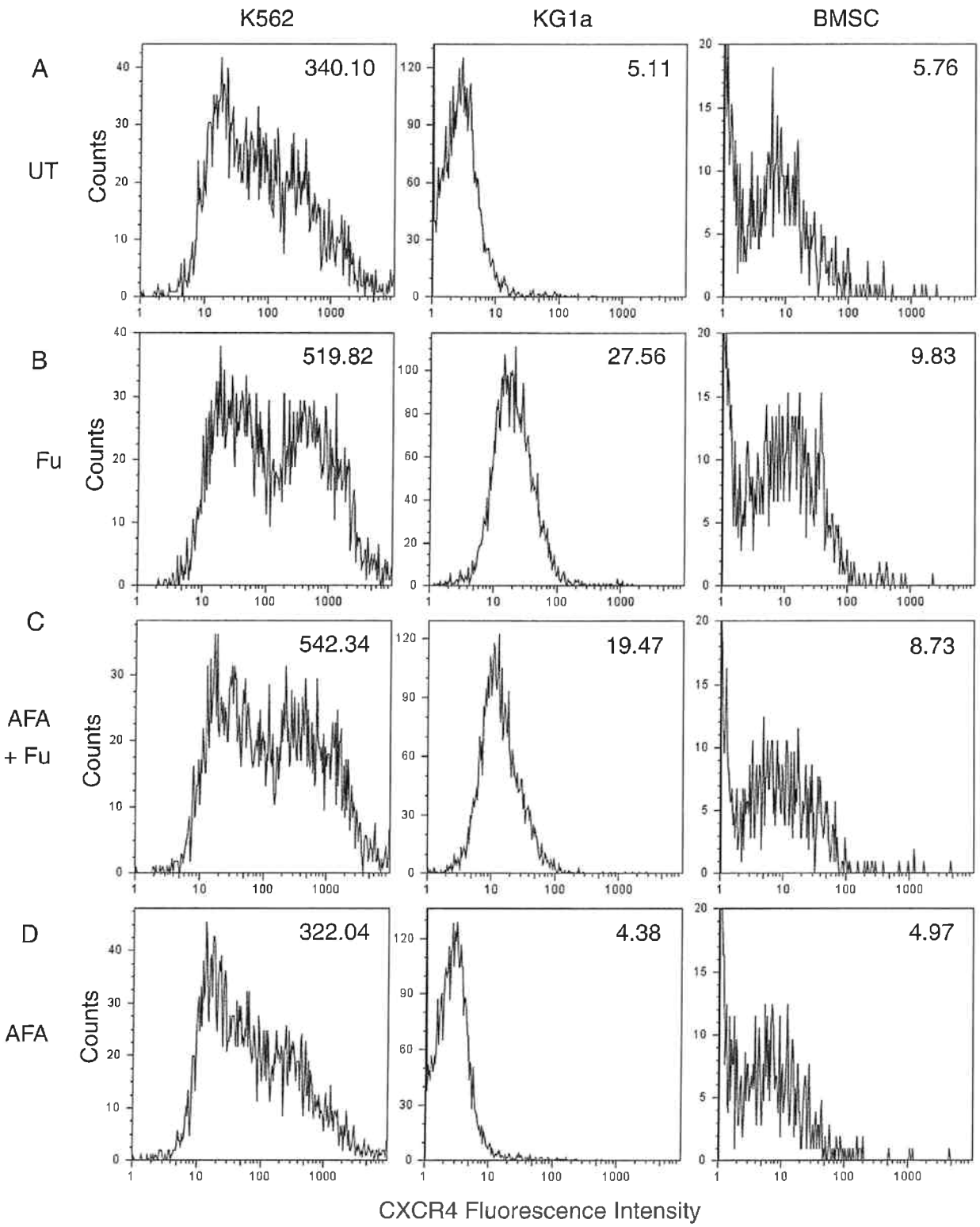

Fig. 3. Treatment of BMSC and the cell lines KG1a and K562 with the LSL fucoidan (Fu) resulted in rapid externalization of the chemokine receptor CXCR4, as measured by immunostaining and flow cytometry. Row A shows the baseline expression of CXCR4 on UT cells. Row B shows the level of CXCR4 expression after treatment with Fu. Row C shows the inhibition of CXCR4 expression on KG1a and $CD34^+$ BMSC cells where both Fu and AFA-W were added simultaneously. Row C also shows that the effect did not extend to the $CD34^-$ cell line K562. The last row D shows that AFA-W alone did not trigger CXCR4 expression on the cell surface. The data shown are representative of testing involving three different bone marrow samples and three separate experiments involving the two cell lines.

selectin/IgG1 F_c fusion protein, we captured an L-selectin-binding compound from the AFA water extract (AFA-W). Under reducing conditions, this compound showed a distinct double band. Two proteins had apparent molecular weights at 57 and 54 kDa, respectively (Fig. 1). The native protein is larger and estimated to be of an approximate molecular weight of 160–180 kDa.

Comparing band density to SDS gel electrophoresis of a standard curve of known amounts of bovine serum albumin, we estimated that the ligand is present at 0.2 μg/g of dried AFA biomass (data not shown). The molecular weights of the two subunits of the AFA-LSL were present in equal amounts, as estimated using scanned gels from a series of experiments.

3.2. AFA-W specifically reduces TQ1 immunostaining of L-selectin on human PMN cells

The incubation of PMN with AFA-W resulted in reduction of immunostaining with the TQ1 anti-human L-selectin monoclonal antibody, which is known to be specific for the ligand-binding area of L-selectin [32]. On PMN, an

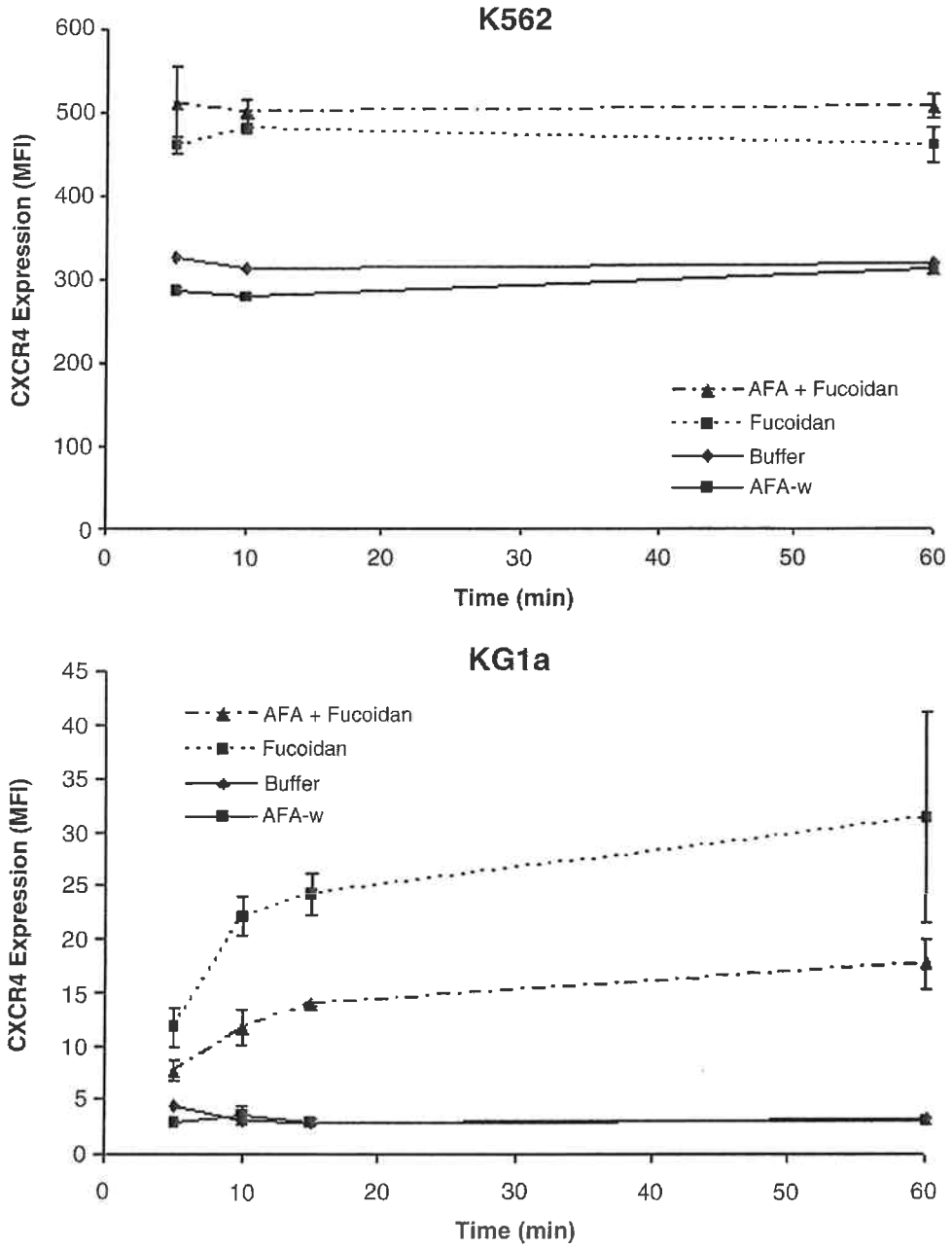

Fig. 4. Time course evaluation of CXCR4 expression on K562 and KG1a cells was performed by immunostaining and flow cytometry after the cells were treated with Fu, AFA-W, or a mixture of both Fu and AFA-W. Treatment with Fu led to an immediate (5 min) and sustained (1 h) increase in CXCR4 expression on both cell types. Incubation of the cells with a mixture of Fu and AFA-W resulted in a significant reduction of CXCR4 expression on the KG1A, but not K562, cells, indicating a competition between Fu and the AFA-derived LSL on KG1a cells.

approximate 50-fold reduction in TQ1 staining was seen when cells were preincubated with AFA-W (Fig. 2). The AFA-W-mediated reduction of TQ1 staining was strongest on lymphocytes and PMN but was also observed on monocytes (data not shown). The expression of CD11b was slightly up-regulated, while no significant changes were observed for other adhesion markers (CD11a, CD18, CD29, CD49d, CD49e and CD44; data not shown). Formalin-fixed peripheral blood lymphocytes were incubated in the absence or presence of serial dilutions of AFA-W. Staining of lymphocytes with the TQ1 antibody showed a dose-dependent reduction in TQ1 binding to L-selectin with increasing concentrations of AFA-W. As the effect was seen also on the formalin-fixed lymphocytes, the reduced staining could not be due to shedding of L-selectin but was indeed a result of a direct binding to the ligand-binding area.

3.3. AFA-W inhibits the fucoidan-induced CXCR4 expression on $CD34^+$ cells from bone marrow and on the KG1a $CD34^{bright}$ cell line but not on the $CD34^-$ cell line K562

CXCR4 expression was evaluated on $CD34^+$ cells from human bone marrow. The $CD34^+$ cells from bone marrow responded to fucoidan by increasing the expression of CXCR4 from intracellular stores (Fig. 3A, right column of histograms). Fucoidan-induced expression of CXCR4 receptors was partially inhibited by AFA-W.

In addition to the $CD34^+$ BMSC, the two primitive cell lines KG1a and K562 were compared in terms of responsiveness to L-selectin ligation by fucoidan and the ability of AFA-W to inhibit this response. Both cell lines are brightly positive for L-selectin, as evaluated by staining with the TQ1 monoclonal antibody. KG1a is brightly positive for CD34, whereas K562 is further differentiated; it is negative for CD34 and positive for GlyA due to its commitment to the erythromyeloid lineages. Both cell lines contain intracellular reservoirs of the CXCR4 chemokine receptor, as revealed by intracellular staining for CXCR4 (data not shown), but only the KG1a cell line responds to L-selectin ligation by externalizing this receptor (Fig. 3A, left and center columns). AFA-water extract is able to block the fucoidan-mediated effect on CXCR4 expression on KG1a.

The time course for the fucoidan-induced CXCR4 expression on KG1a and K562 is shown in Fig. 4. The inhibition of CXCR4 expression by AFA-W was effective across the time course and was statistically significant ($P_{15\ min}<.02$).

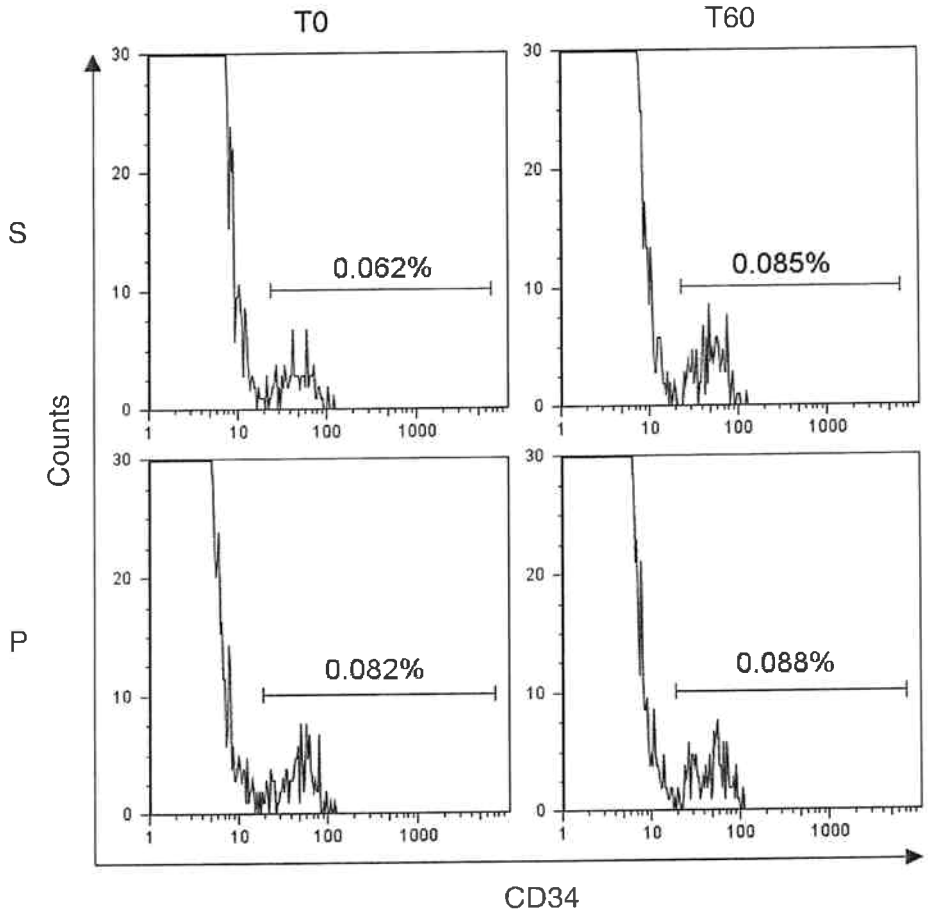

Fig. 5. Histograms showing the percent $CD34^+$ lymphocytes immediately before consumption (T0) and at 60 min after consumption (T60) of either StemEnhance (S) or placebo (P). The data shown represent one of the 12 study participants.

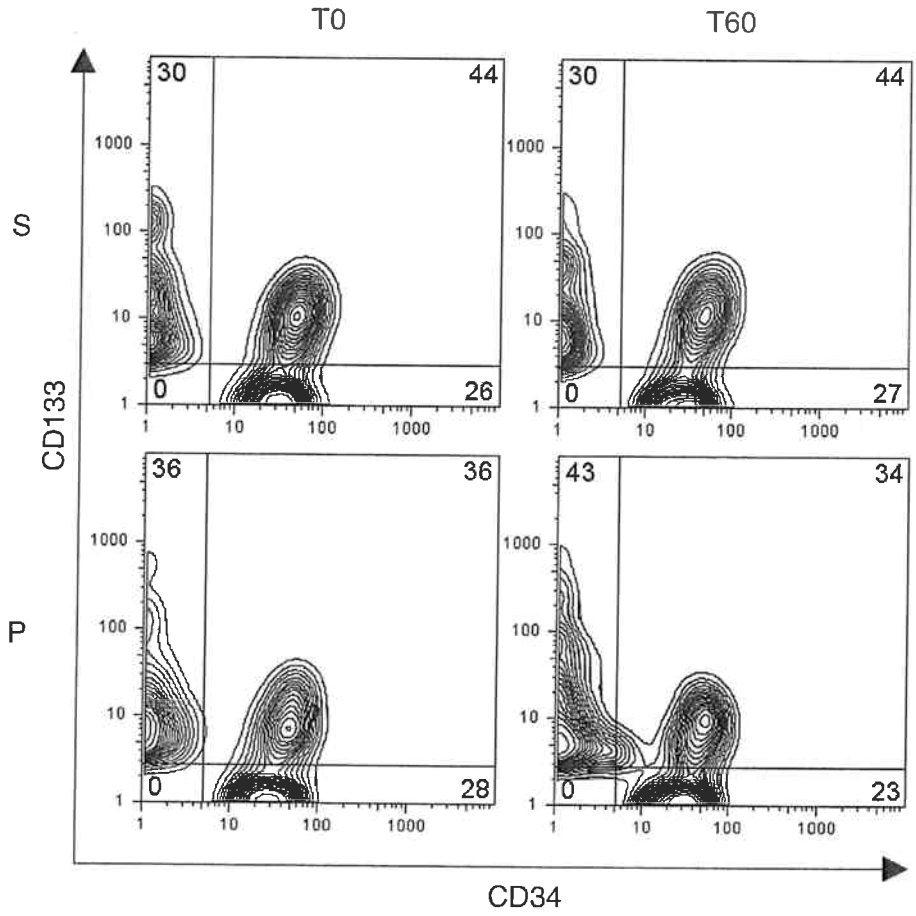

Fig. 6. Contour plots showing the relative distribution of $CD34^+$ $CD133^-$, $CD34^+$ $CD133^+$, and $CD34^-$ $CD133^+$ cells immediately before consumption (T0) and at 60 min after consumption (T60) of either StemEnhance (S) or placebo (P). The data shown are representative of one of the 12 study participants.

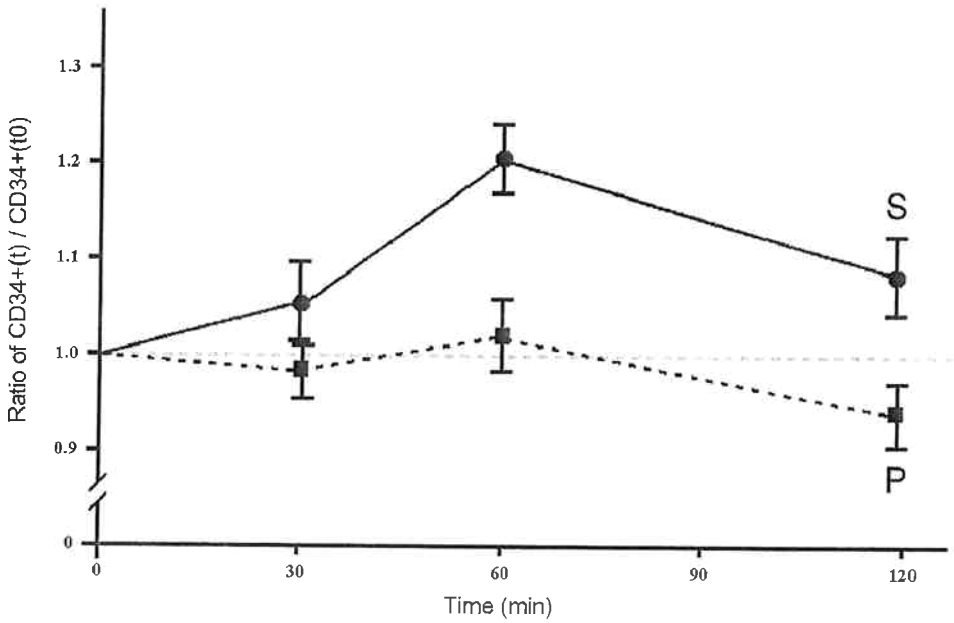

Fig. 7. Consumption of StemEnhance triggered a transient increase in the number of circulating $CD34^+$ cells that peaked at 60 min after consumption (25±1%). The data shown are the averages of changes within each group and were calculated in the following manner: the actual numbers of $CD34^+$ cells were calculated by multiplying the percent $CD34^+$ cells, based on immunostaining and flow cytometry on triplicate samples from each sampling time point, by the number of lymphocytes, as obtained by CBC, also performed in triplicate for each sampling time point, normalized to the value 1.0 at time 0 (immediately prior to consumption). The lines connecting each data set do not indicate a linear function but only serve to indicate which data sets belong to each treatment.

3.4. In vivo: consumption of an AFA extract rich in AFA-LSL resulted in a transient increase of circulating CD34$^+$ cells

The level of circulating CD34$^+$ stem cells was compared before and after ingestion of 1 g of the AFA-LSL-rich extract StemEnhance or placebo. The staining included both CD34-FITC and CD133-PE on the PBMC, and analysis was performed on CD34$^+$ overall in parallel to CD34$^+$ CD133$^-$, CD34$^+$ CD133$^+$ and CD34$^-$ CD133$^+$. The change in percent CD34$^+$ lymphocytes from T0 to 60 min after consumption (T60) of either StemEnhance or placebo are shown for 1 out of the 12 study participants (Fig. 5).

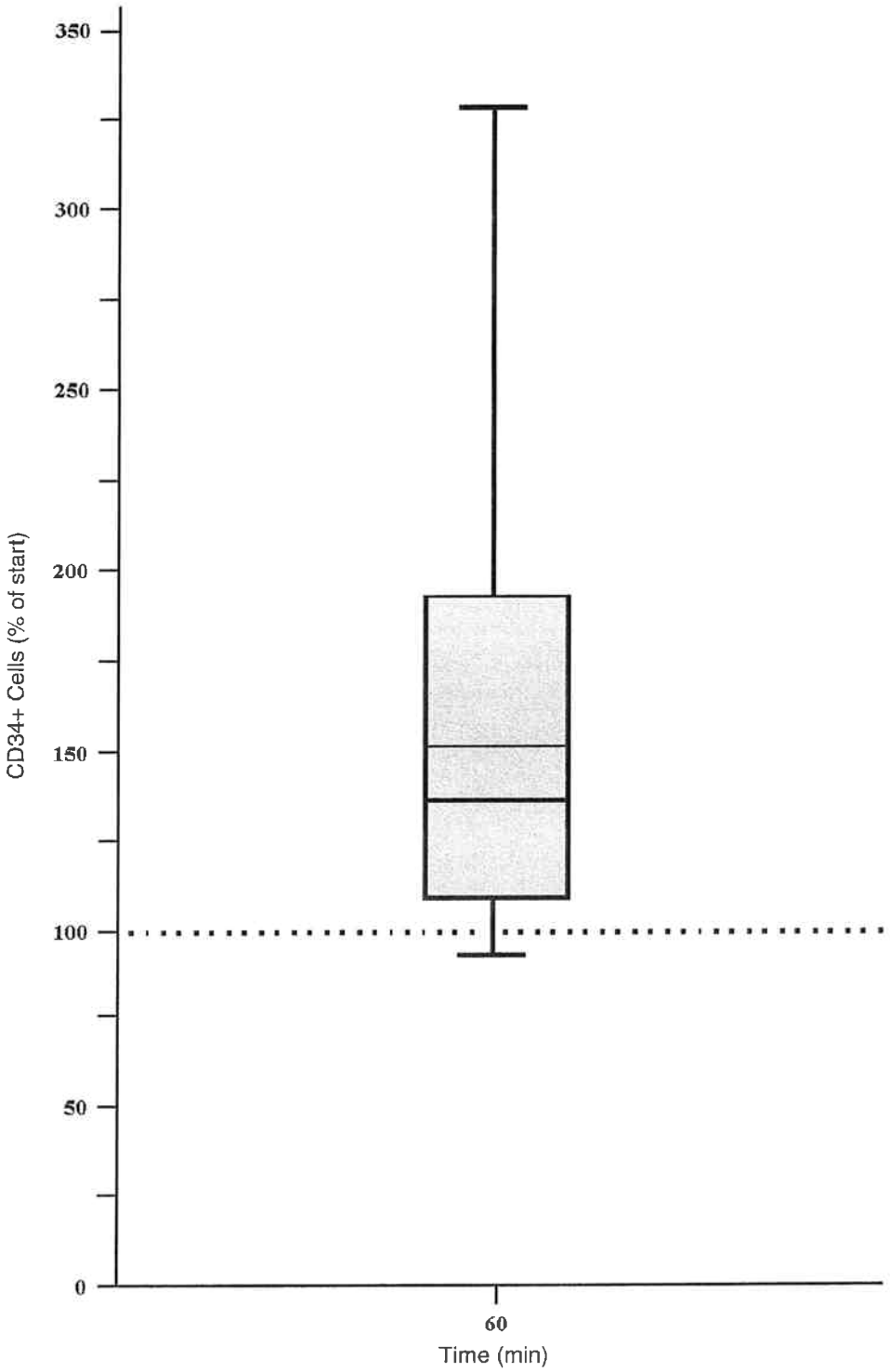

Fig. 8. Multiple testing on one individual on 16 different test days revealed a similar response to StemEnhance on different test days. However, the magnitude of the response varied between tests. The box plot shows the 25–75% spread of the results, the median (M), the average (A), and the lowest and highest response.

Table 1
Lack of effect of StemEnhance on the level of white blood cells and the lymphocyte subset

	StemEnhance				Placebo			
Time (min)	0	30	60	120	0	30	60	120
WBC	100	104±1	104±1	109±5	100	107±3	104±3	111±4
Lymphocytes	100	107±2	108±2	118±5	100	109±3	109±3	124±5

WBC, white blood cells.

Only the analysis of $CD34^+$ showed a significant difference upon consumption of StemEnhance. The relative distribution of CD34 vs. CD133 on the lymphocyte population, gated to exclude any cell that did not express either stem cell marker, is shown in Fig. 6. The proportion of $CD34^+$ $CD133^+$ vs. $CD34^+$ $CD133^-$ cells remained constant, indicating that the mobilized progenitor cells included cells of both phenotypes.

When including all volunteers, ingestion of StemEnhance resulted in an 18±3% increase in the number of circulating $CD34^+$ cells, maximizing around 60 min after ingestion ($P<.0003$). This was in contrast to placebo, which resulted in only minor fluctuations of the levels of $CD34^+$ cells in the blood circulation over 2 h. Questionnaires completed by the volunteers on every experimental day revealed that three of the volunteers met criteria for exclusion (e.g., significant lack of sleep, severe anxiety) on at least one experimental day. Exclusion of these volunteers in the analysis resulted in a 25±1% increase in the number of circulating stem cells at 60 min ($P<.0001$) (Fig. 7). As expected a priori, effects for people (ID), the interaction between people (ID), and the interactions between people and treatment type (StemEnhance or placebo) and people and time differed significantly. There was no significant difference between replicate analyses, nor the interactions between replicate analyses and treatment type (StemEnhance or placebo) and replicate analysis and time. That is, the analytical variability was similar across people, treatment types, and times. Also, overall analysis on normalized data for each study participant showed significance.

In order to test the repeatability of the effect of consumption of StemEnhance on the levels of $CD34^+$ cells in the peripheral blood, 16 separate experiments were performed on one volunteer. The average increase in the number of circulating stem cells was 53±16%, with a median of 36% and a highest and lowest increase of 233% and −4%, respectively (Fig. 8).

No statistically significant changes were observed when comparing numbers of total leukocytes or lymphocytes between the two treatments (StemEnhance vs. placebo) (Table 1).

4. Discussion

Dietary strategies for supporting stem cell biology represent an emerging field of nutritional medicine. The understanding of the effect of nutrition on stem cells needs to include stem cell viability, proliferation, mobilization, and tissue-specific homing. It has been reported that *Spirulina* consumption may increase erythropoiesis in a mouse model [33]. In addition, antioxidant-rich blueberry and green tea extracts have been reported to increase stem cell life span and proliferation in vitro [34], which may provide a better understanding of some aspects of antioxidant therapy in aging.

This study involving human stem cell mobilization was triggered by a few cases of empirical evidence that consumption of an extract from AFA, enriched for the LSL, resulted in unexpected extent of recovery after traumatic injuries to the central nervous system. The mobilization of stem cells is complex but involves two key features: (1) interference with the adhesion of stem cells to the bone marrow via L-selectin and (2) a reduction of the chemotactic response to SDF-1 via the CXCR4 chemokine receptor. We found that the cyanobacterium AFA contains a novel compound that specifically binds to the ligand-binding area of human L-selectin. It is composed of two subunits with apparent molecular weight around 54–57 kDa under reducing conditions. This compound differs from the 100,000 kDa polysaccharide from AFA previously described [35]. This ligand for human L-selectin, precipitated from AFA water extract (AFA-W), was able to modulate the functional response on human lymphocytes in vitro and interfered with the up-regulation of CXCR4 when bone marrow stem cells were exposed to another LSL, fucoidan. In parallel to human bone marrow stem cells, the primitive $CD34^{bright}$ KG1a cell line was responsive to L-selectin-mediated up-regulation of CXCR4, possibly due to its stage of differentiation being comparative to a subset of bone marrow-resident stem cells. The ability of AFA-W to down-regulate the expression of CXCR4 on BMSC and KG1a, but not K562, suggests that this ligand could play a role in stem cell mobilization from the bone marrow.

This may specifically have relevance for mobilization of stem cells from marrow into the circulation, as it has previously been shown that interference with the SDF-1/CXCR4 axis is a primary mechanism of stem cell mobilization from the bone marrow [11]. The LSL may also have a direct effect on stem cell release, as LSLs have been proposed as a therapeutic method for stem cell release and increase of the number of circulating stem cells [29].

A double-blinded placebo-controlled crossover study involving 12 healthy subjects showed that consumption of an AFA extract enriched in this ligand (StemEnhance) resulted in a small but significant increase in the number of

circulating $CD34^+$ stem cells, peaking at 1 h after consumption. The effect was statistically significant ($P<.0001$). When tested on one individual on many occasions, the increase in the number of circulating stem cells after consumption of StemEnhance averaged $52\pm16\%$ and varied greatly from 96% to 333% of baseline value. Interestingly, the average response in the one individual tested repeatedly on 16 different study days, and the average response to StemEnhance in the double-blind randomized study involving 12 people was similar, with an increase in $CD34^+$ cells at 153% vs. 125%, respectively. The hypothesis that StemEnhance transiently increases the levels of circulating $CD34^+$ cells is supported by significance for the difference between the two treatments and the interactions of this difference with person and time. This suggests a significant consistency in the response, despite day-to-day fluctuations, which may have contributed to an underestimation of the response to StemEnhance in the double-blind study.

The increase in the number of circulating $CD34^+$ cells peaked within 1 h after consumption of StemEnhance. This is in contrast with the response time seen with the known mobilizer granulocyte colony-stimulating factor (G-CSF), the response of which peaks after a few days of injection [36,37]. It is believed that G-CSF triggers stem cell mobilization by activating proteolytic activity in the marrow, which degrades SDF-1, interfering with the SDF-1/CXCR4 axis [11]. More comparable to StemEnhance is the response to the CXCR4 antagonist AMD3100 that peaks around 6 h after injection [38]. This supports the view that the effect of StemEnhance on stem cell mobilization may be caused by its LSL, down-regulating the expression of CXCR4. The magnitude of the mobilization obtained with StemEnhance (18–25%) is much smaller than what is seen with G-CSF and AMD3100 (20- to 200-fold [36,37]). Recent studies using G-CSF and AMD3100 have added evidence for the potential role of stem cell mobilizers in the mitigation of various diseases such as cardiomyopathies [39,40], kidney failure [41], multiple sclerosis [42], stroke [43,44], wound healing [45], as well as many other health conditions [46]. Such compounds, however, can only be used for short periods of time due to severe side effects [28]. However, such an extreme increase in the number of circulating stem cells may not be required to achieve health benefits. Tomoda and Aoki [47] quantified the level of circulating stem cells in victims of acute myocardial infarction and reported that individuals with more stem cells showed greater recovery of ejection fraction 6 months after the incident. Werner et al. [48] related the levels of circulating stem cells with the risk of cardiovascular incidents in 519 patients with coronary artery disease and concluded that the level of circulating $CD34^+$ endothelial progenitor cells predicted the occurrence of cardiovascular events and death from cardiovascular causes.

Two recent publications have further confirmed the association between G-CSF-mediated stem cell mobilization after acute myocardial infarction (AMI) and improved cardiac repair. The degenerative remodeling of the heart often seen over time after AMI was prevented by G-CSF treatment, as long as percutaneous coronary intervention (PCI) was performed early rather than late [49]. G-CSF treatment provided a significant increase in the short-term myocardial perfusion [50]. The conclusions presented from these two trials are different from a Korean study in which G-CSF-mediated mobilization alone had little effect on increased cardiovascular output [51]. The Korean study found greater effect when intracoronary injection with stem cells was performed in conjunction with G-CSF treatment. It is important to note the different timing of G-CSF treatment in relationship to the PCI procedure when comparing these two studies. In the German study protocol, the PCI was performed first and then followed by G-CSF injections, and the G-CSF injections were started within 90 min after PCI. In the Korean study protocol, an initial 4-day course of G-CSF injections were followed by the PCI, and no further G-CSF treatment was given after the PCI procedure was completed. The relative simplicity of G-CSF injections as a singular treatment option makes this an attractive option if proven to provide significant benefit to the patients. In addition to further understanding the consequences from the two different study protocols, it would also be interesting to further evaluate whether geographical differences exist in the underlying causes of cardiac function, including diet- and stress-related factors, as well as additional differences in medical management of cardiac disease prior to and during myocardial infarct between studies.

At this point, the effect of a massive but transient increase in the number of circulating stem cells, as induced by G-CSF injection, has not been compared to the effect of a mild but daily increase in the number of circulating stem cells, such as what is seen after consumption of StemEnhance. The 25% increase in the number of circulating stem cells occurring after ingestion of 1 g of StemEnhance may have positive effects on various health conditions and, when triggered daily for several weeks or months, might contribute to cardiac tissue regeneration. Studies should be conducted to investigate the potential of such approach for therapeutic purposes.

The observation that the mobilized progenitor cells included cells of both the $CD34^+$ $CD133^+$ and $CD34^+$ $CD133^-$ phenotypes may indicate that a broader range of different types of progenitor cells are affected, instead of a single type or developmental stage of stem cells. Circulating $CD133^+$ cells are reported to include endothelial progenitor cells, which play a role in endothelial repair. The study by Engelmann et al. [50] showed that the phenotypes of G-CSF-mobilized stem cells contributing to this improvement included $CD31^+$ $CD34^+$ $CD117^+$ $CD133^+$ cells. In patients with coronary heart disease, a reduced number of circulating $CD133^+$ cells has been proposed as an independent risk factor for erectile dysfunction [52]. In particular, our data raises the question

as to whether daily consumption of StemEnhance may counteract the reduced number of $CD133^+$ cells in the circulation of patients with cardiovascular disease linked to endothelial dysfunction, including erectile dysfunction. Empirical observations suggest that consumption of StemEnhance for longer periods of time might indeed bring significant improvement in various health conditions, including specific neurodegenerative diseases, chronic obstructive pulmonary disease, kidney insufficiency, and other degenerative problems. However, rigorous studies are necessary to examine the effects of StemEnhance on specific degenerative diseases.

In conclusion, our data presents the observation that consumption of an herbal extract can significantly alter the proportion of stem cells in the circulation. The novel LSL isolated from AFA has complex biological activities in vitro that could explain the increase in circulating stem cells observed after consumption of the AFA extract StemEnhance in vivo. The extent to which this LSL may be responsible for the in vivo effect on stem cell mobilization and the effect of such mobilization on various health conditions are currently subject for further study.

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