The Nutraceutical Alpha Lipid Colostem™ Modulates Peripheral Blood and Bone Marrow Stem Cell Subsets following Oral Supplementation

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Abstract: Alpha Lipid Colostem™ is a proprietary blend of colostrum and other natural ingredients, designed to enhance stem cell activity following consumption. Previous animal feeding studies demonstrated that oral supplementation increased the frequency of hematopoietic stem cells in the bone marrow. To further characterise the steady-state effect of this supplement on stem cell activity, a feeding study was conducted in groups of mice (n=5 per group) to investigate circulating as well as bone marrow hematopoietic and mesenchymal/stromal stem cell subsets using multi-colour flow cytometry-based methodologies. The results demonstrated that supplementation increased the number and proportion of hematopoietic stem cells (HSC) in the blood, suggesting there was effective release of stem cells from the bone marrow niche into the circulation. Analysis of bone marrow cells showed a concomitant reduction in the proportion of total HSC, however within the HSC compartment there was a relative increase of $CD34^+c\text{-}Kit^+$ and reduction in $CD34^+Sca\text{-}1^+$ HSC subsets. Mesenchymal/stromal stem cell ($CD105^+CD29^+$) numbers showed a decrease in the peripheral blood, with an increased proportion detected in the bone marrow. Alpha Lipid Colostem supplementation also correlated with increased the number of blood granulocytes demonstrating enhancement of hematopoietic stem cell differentiation. These findings further strengthen the hypothesis that dietary supplementation with a synergistic combination of bovine colostrum and immune stimulatory carbohydrates promotes coherent activation of the stem cell niche.

Keywords: Alpha Lipid Colostem™, bone marrow stem cell niche, circulatory stem cell pool, feeding studies, food supplement, hematopoietic stem cells, mesenchymal/stromal stem cells, mobilization.

INTRODUCTION

Activation of the bone marrow, a rich source of endogenous stem cells, is central to ongoing homeostatic tissue repair pathways [1-3]. Hematopoietic stem cells (HSC) are renowned for their role in replenishing the immune system and contributing to tissue repair [4]. Mesenchymal/stromal cells (MSC) are a distinct stem cell subset increasingly recognised for not only their ability to differentiate into a diverse range of cell types such as bone, cartilage and muscle [5], but also for their natural immune regulatory properties and ability to dampen inflammation at the site of tissue injury [6]. This activity is also beneficial for creating a microenvironment that supports stem cell recruitment, engraftment and differentiation into new tissue-committed cells. In addition, MSC are known to secrete paracrine factors including cytokines and growth factors that promote cell survival, as well as neovascularisation [7], all which also contribute to the wound healing response [5, 8].

Whilst they are distinct progenitor cell types, HSC and MSC share the bone marrow stem cell niche and act in concert with each other. MSC have been shown to interact with HSC to stimulate and enhance their proliferation through their influence on the microenvironment, and as such play a role in regulating hoaematopoiesis. In this way, MSC contribute to the hematopoietic stem cell niche and maintain the stem-ness of HSC, and it is likely both stem cell types are important for maintaining tissue homeostasis [9]. For these cells to be effective it is essential that they can migrate from the stem cell niche and circulate to the site of damage where they then act locally following extravasion in response to signals from damaged tissue [10-14]. Thus the circulation of these cells is important in maintaining a pool of stem cells in distant parts of the body as well as responding to tissue injury [9, 14].

The ability to manipulate the immune system to increase circulating stem cell numbers has already been validated in the clinical setting, where the number of stem cells in peripheral blood can be increased by protocols using hematopoietic cytokines that are known to activate the release of bone marrow stem cells into the circulation [15, 16]. Therefore, in addition to improving clinical outcomes from stem cell-related therapies, developing ways to manipulate bone marrow stem cells are likely to be of benefit for maintaining a healthy number of circulating stem cells and supporting homeostatic repair mechanisms.

Alpha Lipid Colostem™, a combinatorial immune stimulatory nutraceutical was developed to stimulate stem

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cell release. It comprises natural inducers of hematopoietic activity, as well as growth factors contained within the bovine colostrum component. Initial studies demonstrated that 14 day oral delivery of this formula resulted in increased bone marrow colony forming units indicating that oral Alpha Lipid Colostem could trigger a systemic hematopoietic effect leading to expansion of the bone marrow stem cell niche [17]. The aim of this study was to further determine the effects of oral Alpha Lipid Colostrum on stem cell activity by analysing circulatory as well as bone marrow hematopoietic and stromal cell subsets using multi-colour flow cytometry methodology.

MATERIALS AND METHODS

Animals

Female CD-1 mice (12 weeks old) were used for this feeding study. The animals were maintained according to the principles and guidelines of the ethical committee for animal care at Auckland University. The University of Auckland ethics approval number for this study is C877.

Alpha Lipid Colostem

Alpha Lipid Colostem was obtained from New Image Group, NZ. This is a novel formulation comprising lipid coated bovine colostrum and immune stimulatory carbohydrates, as previously described [17]. The maximum amount of supplement administered (high dose) equated to a total 340 mg of ingredients suspended in water. This was diluted two fold to obtain the low dose of 170 mg of ingredients. The relative amounts of individual ingredients are shown in Table **1**.

Animal Feeding Study

Mice were randomly divided into treatment groups by the animal care facility (5 per group). Alpha Lipid Colostem (1 mL) was administered daily for 14 days between 12-4 PM. Water was administered as a control. An oral gavage needle was used to administer the formulation. At all other times mice were fed *ad libitum* on standard mouse chow. On day 14 of the study, heparin anti-coagulated peripheral blood was sampled *via* the tail vein for determination of stem cells in the peripheral blood by multi parametric flow cytometry analysis. Mice were then euthanized and bone marrow was harvested. A bone marrow cell suspension for flow cytometry analysis was prepared using both femurs from each animal using standard techniques. Each mouse was analyzed individually.

Table 1. Composition of alpha lipid colostemtm (ALC) doses administered by oral gavage, daily for 14 days. doses were administered in 1 mL and water was used as a control.

Ingredient	ALC top mg/dose	ALC Bottom mg/dose
Alpha Lipid Colostrum	192.0	96.0
Scuterllaria	46.9	23.4
Fructus Jujube berry	46.9	23.4
Turmeric Extract	23.4	11.7
Yeast Extract	11.7	5.9
Fucoidan (seaweed extract)	9.4	4.7
Carnosine	9.4	4.7
Total	339.7	169.8

Flow Cytometry Analysis

Peripheral blood samples were stored at room temperature and bone marrow cells on ice until analysis. Cell staining (100 μL of blood/test; 100 μL of bone marrow suspension) with panels of fluorescent monoclonal antibodies to detect stem cell subsets was performed. Peripheral blood samples were stained in TruCount™ tubes (Becton Dickinson), which contain a known number of fluorescent counting beads, using a lyse-no wash method to facilitate accurate enumeration. Red blood cells were lysed following staining using FACSlyse (Becton Dickinson). Bone marrow cells were not stained in TruCount tubes since the absolute number of bone marrow cells obtained from each mouse would not be equivalent. Approximately 500,000 events were acquired from each tube. Fluorescent antibody staining panels (all from Biolegend, USA) used were as follows: For hematopoietic stem cells: Lineage-Brilliant violet, CD34-PE, CD117 (c-Kit)-APCCy7, Sca1-PerCPCy5.5, CXCR4-AF647. For mesenchymal/stromal cells: Lineage-PE, CD105-APC, CD29-PECy7 and Hoechst nuclear dye. Samples were analyzed on a LSR II flow cytometer (Becton Dickinson). Stem cell subsets and fluorescent TruCount beads were resolved using multi-gate analysis (FlowJo), and the absolute counts of each subset were determined relative to the known number of TruCount beads. Flow cytometry gating strategies were defined in a sample from the high dose group. These gating algorithms were then used for automatic analysis of the remainder of the data set.

Statistical Analysis

All data were analyzed using Prism GraphPad (La Jolla, CA). Two group comparisons were assessed by Student's t test (non-parametric). P values < 0.05 were considered significant.

RESULTS

$CD34^{+}Lineage^{-}$ hematopoietic stem cells were identified according to the sequential gating strategy shown in Fig. (**1**) and absolute counts determined based on numbers of stem cells (P5) relative to TruCount beads (P2). Supplement fed mice showed a significant increase in the number of circulating hematopoietic stem cells, which was of a similar magnitude for both doses of supplement. This increase was also reflected in the proportional increase of $CD34^{+}$ cells relative to total blood leucocytes (Fig. **1b**). In contrast, mesenchymal/stromal cells identified based on CD29 and CD105 staining did not show significantly different circulating numbers (data not shown). This may be due to the fact that they are very rare subsets in the peripheral blood

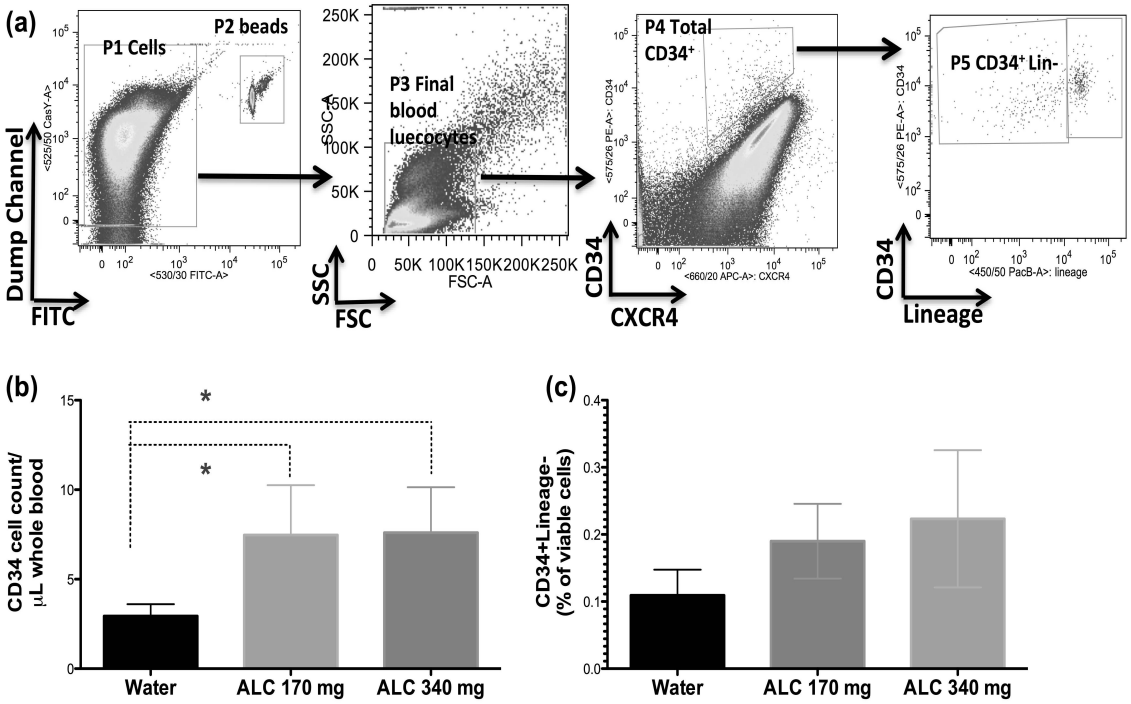

Fig. (1). Flow cytometry quantification of peripheral blood $CD34^+$ Lin^- hematopoietic stem cells. Whole mouse blood was stained with hematopoietic stem cell cocktail in TruCount tubes (Materials and Methods). $CD34^+$ Lin^- cells were gated according to sequential gating (**a**): Cells not beads (P1) AND leucocytes by scatter (P3) AND total $CD34^+CXCR4^+$ (P4) AND $Lineage^-$ (P5). (**b**) The absolute number of circulating $CD34^+$ Lin^- was determined relative to the known concentration of the TruCount beads (P2) (**c**) $CD34^+$ Lin^- cells were also calculated as a proportion of total viable leucocytes (P3).

and difficult to resolve in small whole blood volumes as were used in this study.

Since $CD34^+Lin^-$ stem cells originate from the bone marrow, we analysed bone marrow cells using the same panel of fluorescent antibodies to determine any correlative changes for supplement-mediated effects on hematopoietic as well as stromal stem cell subsets. Overall levels of cell proliferation were determined by Hoechst DNA-specific dye fluorescence, where cells in the S/G2 phase of the cell cycle show increased Hoechst fluorescence (Fig. **2a**). In agreement with previous findings [17], Alpha Lipid Colostem supplementation was associated with an increased proportion of dividing cells (Fig. **2a**), and confirmed that the bone marrow had been stimulated in this study. Analysis of the proportion of $CD34^+$ stem cells in the bone marrow showed a modest decrease in this population in Alpha Lipid Colostem fed animals, with similar levels of change relative to water fed animals detected at both supplement doses, although only the 170 mg group reached statistical significance (Fig. **2c**). Such a decrease in bone marrow levels appears to coincide with the increased number and proportion of $CD34^+$ cells detected in the peripheral blood, suggesting that factors promoting release of stem cells from the bone marrow niche are being induced at effective levels.

To describe the effect of supplementation on the bone marrow stem cell niche further, $CD34^+$ cell associated Sca-1 and c-Kit co-expression patterns were analysed. Stem cell marker c-Kit defines a subset of $CD34^+$ progenitor cells that have been particularly associated with haematopoiesis [18]. The proportion of c-Kit^+ co-expressing $CD34^+$ cells (Fig. **2c**) significantly increased in both of the supplemented groups (Fig. **2d**). Conversely the proportion of $CD34^+$ cells co-expressing high levels of Sca-1 antigen (Fig. **2e**) were notably absent in supplement fed groups suggesting their preferential emigration (Fig. **2f**).

Bone marrow cells expressing mesenchymal/stromal cell markers were also determined. Cells lacking lineage antigen expression but expressing $CD105^+$ antigen have been designated mesenchymal stem cells (MSC) by others [19] and these markers were used to identify mesenchymal cells in the lineage negative bone marrow mononuclear fraction (Fig. **3a**). To further delineate $CD105^+$ cells, co-expression of another stromal/mesenchymal marker, CD29 was determined (Fig. **3a**). CD29 is an β-1 integrin adhesion molecule that has been shown to be highly expressed on freshly isolated human mesenchymal stem cells [19]. CD29 plays an important role in MSC migration and adhesion at the site of tissue damage. We detected a clear population of $CD105^+CD29^+$ cells in the bone marrow Lin^- progenitor cell subset (Fig. **3a**), and whilst statistical significance was not reached ($p=0.076$), the data indicated a trend for a higher frequency of these cells in the high dose supplemented group (Fig. **3b**). Peripheral blood quantification of MSC (Fig. **3c**) showed an opposite response, demonstrating a lower number of circulating $CD105^+CD29^+Lin^-$ cells in the high dose group compared to the low dose group and water fed animals (Fig. **3d**). DNA cell cycle content analysis of bone marrow MSC subset was attempted to determine if there was any enhanced proliferation of these cells, but there were too few events to obtain a reliable DNA content histogram (data not shown).

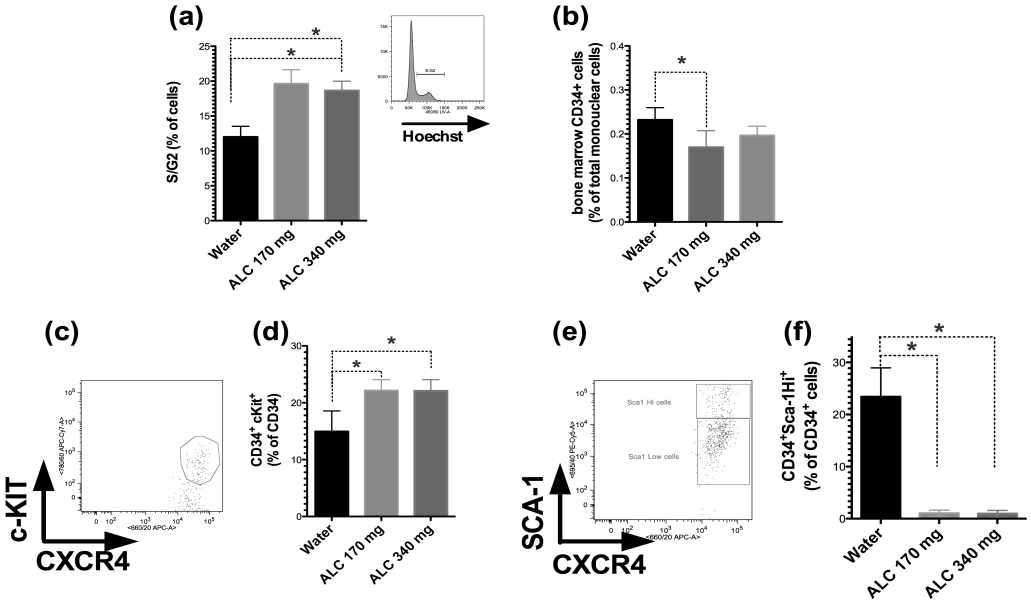

Fig. (2). Flow cytometry quantification of bone marrow $CD34^+$ subset changes following 14 day supplementation with ALC. Bone marrow cells were stained with the hematopoietic stem cell cocktail and Hoechst DNA binding dye (Materials and Methods). Bone marrow $CD34^+$ Lin^- cells were gated according to sequential gating as shown in Fig. (**1**, **a**) Cell cycle of viable bone marrow cells was determined on viable cell gated events. (**b**) The proportion $CD34^+$ bone marrow cells were determined out of total mononuclear cells. (**c**, **d**) c-Kit expression was determined on gated $CD34^+$ $CXCR4^+$ cells. (**e**, **f**) Sca-1 expression was determined on gated $CD34^+$ $CXCR4^+$ cells.

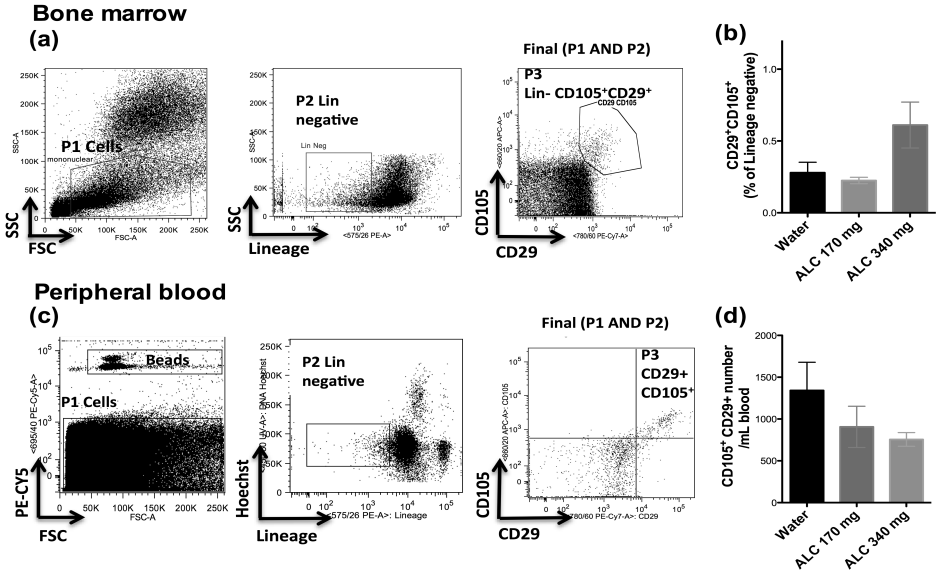

Fig. (3). Flow cytometry quantification of bone marrow and peripheral blood mesenchymal cell subsets following 14 day supplementation with ALC. Bone marrow cells were sequentially gated (**a**) based on light scatter and lineage negative expression (P1 and P2) and the proportion of Lin^- $CD105^+CD29^+$ cells (P3) determined (**b**). Peripheral blood leucocytes and TruCount beads were gated (**c**) and blood MSC isolated based on hoechst nuclear staining for 1N DNA content and negative lineage expression (P1 and P2) and $CD105^+$ $CD29^+$ cells (P3) in the Lin- gate counted. Absolute cell numbers were calculated by reference to TruCount bead number (**d**).

In order to further underscore the impact of supplementation on bone marrow stem cell differentiation, mobilisation of peripheral blood granulocytes was quantified (Fig. **4a**). These cells are known to be released in response to hematopietic immune factors such IL-6 and G-CSF/GM-CSF. Quantification of the absolute count of granulocytes in supplemented animals showed there was a dose-responsive increase in the numbers of blood $lineage^+$ granulocytes. There was also a corresponding increase in the proportion of these cells detected in the bone marrow (Fig. **4b**). These findings demonstrate that Alpha Lipid Colostem is able to effectively stimulate the bone marrow stem cell niche to enhance hematopoietic stem cell differentiation and emigration into the circulation, as well as maintain ongoing replenishment of the bone marrow pool of lineage-committed precursor cells.

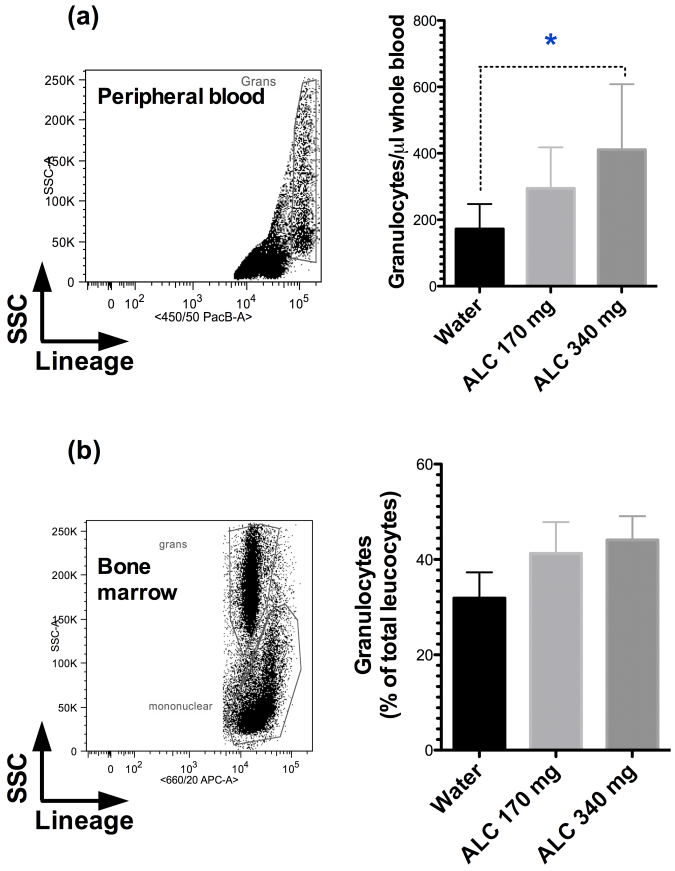

Fig. (4). Flow cytometry quantification of peripheral blood (**a**) and bone marrow granulocytes (**b**) using lineage versus side scatter (SSC) correlation following 14 day supplementation with ALC.

DISCUSSION

The present findings demonstrate that Alpha Lipid Colostem sustained an effective release of hematopoietic stem cells into the peripheral blood following 14 days of supplementation, therefore increasing the number of stem cells in the circulatory pool. Associated with this was a reciprocal reduction of the proportion of bone marrow HSC. The mechanism of this release is likely to depend in part on the ability of Alpha Lipid Colostem to induce GM-CSF and G-CSF *in vivo*, which is in agreement with previously described *in vitro* and *in vivo* activities for this supplement [17]. GM-CSF acts by interfering with SDF-1-CXCR4 ligand-receptor-interactions that maintain stem cells bound to the bone marrow stromal cells. Accordingly, decreasing SDF-1 levels promotes egress of stem cells from the bone marrow niche [15, 20, 21]. Influencing CXCR4 expression directly using a CXCR4 antagonist also augments circulating stem cell numbers and synergises with GM-CSF [22, 23]. The ability of Alpha Lipid Colostem to increase the numbers of peripheral blood neutrophils, which depends on the effects of GM-CSF/G-CSF on hematopoietic stem cell differentiation further underscores the ability of this supplement enhance a full complement of bone marrow stem cell differentiation pathways [24]. Interestingly, with increased numbers of circulatory HSC, there was a preferential reduction in the proportion of bone marrow HSC expressing high levels of Sca-1 antigen. Apart from serving as a conventional stem-cell subset marker, Sca-1 antigen co-expression on HSC subsets has been associated with homing and engraftment ability [25]. Thus the reduction of the Sca-1^{+}Hi subset may indicate that these cells were preferentially mobilised. This result is interesting since Sca-1^{+} stem cells have been associated with immune system reconstitution as well as tissue repair [18, 26].

The effect of supplementation on MSC was less apparent than for HSC subsets, which may be a consequence of the fact that MSC are very rare, or perhaps these cells only have a transient peak mobilization response, which occurred before day 14. Nonetheless, the trend for there being reduced numbers of peripheral blood MSC concomitant with an increased frequency in the bone marrow could be interpreted as MSC trafficking from the circulation into peripheral tissues, retention in MSC niches as well as enhanced homing back to the bone marrow stem cell niche, a property associated with both circulatory hematopietic and mesenchymal progenitor cells [10, 11]. Given there is enhanced bone marrow activity under the influence of Alpha Lipid Colostem-induced immune stimulation [17], and the fact that this study was conducted in healthy mice which therefore have no tissue recruitment signals required for MSC extravasion [27], does not support that there was an increased MSC emigration into the tissues, but may suggest an altered migration/niche retention hypothesis.

Only a limited number of studies have been conducted on the ability of other nutraceutical formulations to act as stem cell mobilizing agents [28-30]. The results from a study conducted in a small number of healthy subjects taking a blue-green algae-based supplement showed that depending on the hematopoietic stem cell subset, peak changes were detected between days 1-7 of a 14 day study, with only changes in hematopoietic $CD34^+$ cells being sustained on Day 14 of supplementation [30]. These data however are only qualitative and the absolute number of circulating HSC was not determined, nor were mesenchymal/stromal cells measured in those studies.

Our findings extend the paradigm for nutraceutical enhancement of stem cell activity, by providing evidence of reciprocity between the bone marrow stem cell niche and the circulatory pool under the influence of a stem cell enhancing supplement. Whilst we show enhancement of circulatory HSC, we also show that there is enhanced bone marrow cell division, which may replenish the stem cell pool [24].

It is widely accepted that stem cells form an integral part of the body's regenerative network [31], and currently there is a world-wide effort to better understand how to manipulate stem cells for therapeutic benefit [32] and sustain this activity as the stem cell niche ages and becomes less effective [32-34]. Accordingly, protocols, which can reliably enhance endogenous stem cell activity by stimulating the host immune system, are likely to offer an advantage to healthy individuals for maintaining effective immune function as well as healthy organs and tissues [35, 36]. Furthermore, the clinical challenges facing successful stem cell transplantation, such as enabling successful migration of adoptively transferred cells, as well as maintenance of regenerative signals required for engraftment and long-term therapeutic benefit [3] also may be overcome by exploiting natural means of stimulating the body's own immune system to coordinate this complex regenerative system [37].

CONCLUSION

The findings from this study provide further evidence that long term oral supplementation with Alpha Lipid Colostem can stimulate sustainable hematopoietic and mesenchymal stem cell related activity. This supports the view that there is merit in developing non-pharmacologic means of enhancing endogenous stem cell pathways.

ABBREVIATIONS

GCSF	=	granulocyte colony stimulating factor
GM-CSF	=	granulocyte/macrophage colony stimulating factor
HSC	=	hematopoietic stem cell
IL-6	=	interleukin-6
MSC	=	mesenchymal stem cell
Sca-1	=	stem cell antigen-1
SDF-1	=	stromal cell derived factor-1
VEGF	=	vascular endothelial growth factor

CONFLICT OF INTEREST

New Image Group Ltd developed Alpha Lipid Colostem and sponsored this study. P. Lehrke was General Manager of Research and Development at New Image Group at the time of the research, and is now an independent product development consultant. G. Webster was remunerated as an independent consultant immunologist for the new product development program**.**

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